CLINICOPATHOLOGIC EVALUATION OF PRO- AND ANTIAPOPTOTIC MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA

A Dissertation submitted

in partial fulfillment of the requirements

for the degree of

MASTER OF DENTAL SURGERY

BRANCH – VI

ORAL PATHOLOGY & MICROBIOLOGY

THE TAMIL NADU DR.M.G.R. MEDICAL UNIVERSITY

Chennai- 600 032.

2010-2013

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled, **"CLINICOPATHOLOGIC EVALUATION OF PRO- AND ANTIAPOPTOTIC MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA"**, is a bonafide and original research work done under the supervision of **DR. I. PONNIAH**, Professor & Head, Department of Oral Pathology, Tamil Nadu Government Dental College & Hospital, Chennai.

I firmly state that I, **DR. B. PRATHANA**, is entirely responsible for any violations (if any) and it does not have any binding on my supervisor.

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This is to certify that **Dr.B.PRATHANA,** Post Graduate student (2010-2013) in the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, has done this dissertation titled, **"CLINICOPATHOLOGIC EVALUATION OF PRO- AND ANTIAPOPTOTIC MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA'',** under my supervision in partial fulfillment of the regulations laid down by the **Tamil Nadu Dr.M.G.R. Medical University**, Chennai - 600 032, for **M.D.S.,** (Branch-VI: **Oral Pathology and Microbiology)** degree examination.

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And

Dr. B.Prathana, aged 27 years currently studying as Post Graduate student in Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as 'the PG student and Principal investigator')

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ACKNOWLEDGEMENT

I thank **Dr. K.S.G.A. NASSER M.D.S.,** Principal, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, for permission, support and encouragement.

I would also like to thank the members of the dissertation screening committee and ethical committee for their valuable suggestions and permission to proceed with the study.

I thank my supervisor, **DR. I. PONNIAH,** Professor and Head, Department of Oral Pathology, Tamil Nadu Government Dental College & Hospital, for his help in the dissertation and overall guidance during my MDS course.

I am grateful to **Dr. R. BHARATHI M.D.S.,** Associate Professor, Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, for her valuable guidance, support and encouragement at various phases of my course.

My sincere thanks to **Dr. S. GNANADEEPAM M.D.S., and Dr.M.P.SUMATHY M.D.S.,** Assistant professors, Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003 for their valuable suggestions, caring, constant encouragement and timely help rendered throughout my course.

I take this opportunity to express my gratitude to my colleagues, especially to **Dr.A.N.ARYA, Dr. JAI SANTOSH MANIKANDAN, Dr.S.KUZHALI AND Dr.MUDASSAR SHARIEF.** I thank all the interns from the department of Oral pathology & microbiology, for their valuable help.

I thank my cousin **DR. NIRANJANI**, my friends **DR. RAMYA** and **DR. RAJIV** for providing me the literature support for this dissertation work.

I thank the lab technicians **MRS.HILDA MARGARET** and **MRS. MEENAKSHI** for their help in the laboratory works.

I wish to thank **MR. S. VENKATESAN**, Statistician, zigmaa.org for helping me with the statistical work of my dissertation.

A special thanks to all my patients for their consent, co-operation and participation in this study.

I dedicate this work to my parents, my sister and my brother for their love, care and prayers to overcome all my hardships and for being a constant support throughout my course.

All glory and honor to **THE LORD ALMIGHTY**, who was my shepherd and who had never let me want. I thank him for everything that he is for me. Nothing compares to the promise I have in him.

ABSTRACT

BACKGROUND:

Apopotosis is determined by the balance between the pro- and anti- apoptotic regulators in a cell. Bcl2 is the principal anti apoptotic regulator and bax is the principal pro- apoptotic factor that heterodimerizes with bcl2 and counteracts its function. The ratio of bcl2/bax is known to influence the prognosis of OSCC and it differs between the histological differentiation types of OSCC.

AIM:

Is to determine whether the ratio of bax and bcl2 expression have any significance with regard to clinicopathological parameters.

OBJECTIVE OF THE STUDY

To evaluate the immunohistochemical expression of Bcl 2 and Bax genes in oral squamous cell carcinoma.

MATERIALS AND METHODS:

This is a prospective study involving 60 cases of histologically proven OSCC, collected between January 2012 and June 2012. The paraffin tissue blocks were prepared, cut and were stained immunohistochemically for bcl2 and bax. Their immunopositivity were then semiquantitatively analysed by giving scores to the percentage of cells stained. The bcl2/bax is then mathematically calculated. This ratio is then statistically analyzed for any significance in relation to age, sex, site, habit and clinical stage of the patient.

RESULTS:

The bcl2/bax ratios for tongue cases were significantly higher than that of the other sites. Also within the tongue cases, the ratios for cases which had trauma as the etiological factor had significantly higher values.

CONCLUSION:

OSCC of tongue is known to have poor prognosis than the other subsites of oral cavity as proved by other studies. Higher Bcl2/bax ratio than the rest of the subsites may be one of the reasons for this. Trauma causes a higher bcl2/bax ratio among the various etiologies causing tongue OSCC.

Key words: Apoptosis, Bcl2/bax ratio, Oral squamous cell carcinoma, Tongue OSCC.

CONTENTS

ABBREVIATIONS

BAX	Bcl2 associated protein
BCL-2	B cell lymphoma 2 protein
OSCC	Oral squamous cell carcinoma

INTRODUCTION

Oral cancer is the eight commonest cancer worldwide, being more common in the south central Asian countries.[1] In India, it is the most common fatal cancer type in men (22.9%) and third most common in women (9.8%). [2]

Oral carcinogenesis is the cumulative effect of events due to the combination of genetic predisposition and exposure to environmental carcinogens.[3] In the case of oral squamous cell carcinoma (OSCC), the major environmental factor is tobacco.[1] Chronic exposure to environmental factors cause genetic alterations like amplification of oncogenes and inactivation of tumour suppression genes in the epithelial cells.[4] These alterations result in autonomous growth / increased proliferation.[4] Such altered cells are normally eliminated by a process called apoptosis. However the evasion of this cell death results in further immortality.[4] Or in other words, all neoplastic evolution requires two important steps, uncontrolled proliferation of cells and suppressed cell death of those cells. The cell death occurs by a programmed process called apoptosis.[5] It is defined as, "A form of cell death designed to eliminate unwanted host cells through activation of a coordinated, internally programmed series of events effected by dedicated set of gene products.''[6]

Apoptosis is controlled by a family of regulatory proteins called ''Bcl2 family of proteins", encoded by the Bcl2 family of genes.[6] This family includes both pro- and anti-apoptotic proteins.[6] The anti-apoptotic proteins are Bcl2 and Bcl-xL and the pro-apoptotic proteins are Bax, Bak, Bad, Bik, and Bid.[6] The relative concentrations of the pro- and anti-apoptotic members would decide the outcome of a cell challenged with an apoptotic stimulus.[7] Bcl2 protein is the founding member of this family. It is encoded by

bcl2 gene located in the chromosome 18q21.[8] Bcl-2 stands for B-cell lymphoma 2, as it was the second member of a range of proteins found associated with the mutations described in follicular lymphomas.[8]

The literature shows a number of studies on apoptosis and its regulators in cancers[9-12] including OSCC.[16-18] Majority of these studies involve Bcl2 and Bax among the apoptosis regulators. In carcinogenesis, bcl2 and bax are considered as the principle anti- and pro- apoptotic regulators[13] because bcl2 confers the pre- and malignant oral keratinocytes immunity from apoptosis.[14] It also interferes with the cancer therapeutic methods by protecting the tumour cells from apoptosis induced by radiation, chemotherapy and growth factor deprivation.[9] In contrast, bax plays the pivotal role in pro-apoptotic regulation by binding with and opposing the action of bcl2.[13,14] Thus, the relative concentration of Bcl2 and Bax determines the fate of a cell's response to an apoptotic stimulus.[13]

The purpose of this study is to evaluate the Bcl2/Bax in conjunction with the clinicopathologic parameters in OSCC to ascertain as to whether this ratio is influenced by the clinicopathologic parameters

AIM OF THE STUDY

Is to determine whether the ratio of bax and bcl2 expression have any significance with regard to clinicopathological parameters.

OBJECTIVE OF THE STUDY

To evaluate the immunohistochemical expression of Bcl 2 and Bax genes in oral squamous cell carcinoma.

REVIEW OF LITERATURE

A. STUDIES ON EPIDEMIOLOGY OF OSCC : DISTRIBUTION, AGE, SEX, SITE

In the systematic review by **Boyle et al in 1990**[19] the literature collected from various worldwide were included. This paper addresses the geographical variations in the incidence of OSCC. Madras and poona were ranked as the second and third as the among the places with highest record of Oral cancer. France was ranked first. Males are affected more commonly than females. The top five cities with highest oral cancer cases in women were all from India and they were Bangalore, Madras, poona, Bombay and Magpur. They also explain the influence of etiological relation of tobacco, betal quid, smoking and snuff.

Sherin et al in 2008[20], studied the changing trends in oral cancer in an instituitutional retrospective study of 206 cases in kerala, India; with an emphasis on the young patients (< 40years of age). The mean incidence during the study period was 7.5%. There was no sexual predilection in patients over 40 years but the younger age group showed a 3.5 higher incidence in males. The more commonest site in the older group was buccal mucosa, followed by tongue and gingiva. In younger age group tongue was the most common site, followed by buccal mucosa, gingiva and floor of the mouth. Moderately differentiated OSCCs formed the major histological grade. In young patients a large percentage of them (34%) showed no habit of tobacco, smoking or alcoholism.

Zini et al in 2009[21], analyzed the distribution and trends in oral cancer among Israeli population from 1970 to 2006 (6577 cases). Among these cases 57.2% were male and 42.8% were females. All the patients were above 55 years and it is suggested as another risk factor maker. Most prevalent oral cancer subtype was squamous cell carcinoma among men above the age of 55 years. Females had a higher incidence of SCC in lateral border of tongue, gums and buccal mucosa. The highest oral survival rate was for the lip, and the lowest was for the tongue and gums.

Sharma et al in 2010[22], studied the recent trends in 80 OSCC patients in western Uttar Pradesh in India. Of them 68.7% were men and 31.2% were women with a male : female ratio of 2.2:1. The majority of the patients were in their 4^{th} and 5^{th} decade. 60% of them were using smokeless tobacco and 36.25% were smokers, using beedi or cigarette. The buccal mucosa was found to be the most common site. This may be because that majority of them are smokeless tobacco users, and buccal mucosa is the subset which is in more contact with it. The least common site was the palate.

Marocchio et al in 2010[23] made a retrospective study on1564 OSCC cases, collected in an institution in sao paulo, over a period of 40 years. Males were more commonly affected, but the male: female ratio decreased gradually over the years. Most patients were in their 5^{th} and 6^{th} decade. The gingiva was the most affected site, but the frequency of lower lip involvement increased in the last time period. Regarding lesion size and duration of symptoms at the time of diagnosis, there was a significant difference between the first and last time periods. Smaller lesions were found and the time of lesion development was shorter in the last few years of the study.

In a study by **Byakodi et al in 2012[24],** in an institution in western Maharashtra, India, 112 OSCC cases were studied. 72.32% were male and 27.67% were female patient. The average age of the patients was 30–70 years. 25 to 30% consumed were either tobacco users or alcoholics. The most common site was lower alveolus followed by buccal mucosa.

B. STUDIES ON HABITS ASSOCIATED WITH OSCC

One of the earliest studies made about the risk factors in oral cancer among Indian population was done by **Wahi et al in 1965[25].** This comprehensive study explains that variations in the incidence and common sites involved by oral cancer among different studies are probably because of the differences in the habitual etiological factors in different parts of the world. This involved 1916 patients from Agra, India. The risk factors for oral cancer included tobacco usage, smoking, alcoholism, malnutrition and poor oral hygiene and syphilis. This paper also explains the correlation between habit and the site involved. Out of 1916 patients, 821 of them had history of atleast one of the habits. Tobacco was used either smokless form (tobacco chewing, betal quid + tobacco) or smoked tobacco (bidi, cigarette etc). 75.32% had habit of chewing tobacco in any form. They are in constant contact with the buccal mucosa or alveologingival sulcus. Hence the commonest site in this study was buccal mucosa. Majority of the smokers had cancer in anterior 2/3rd of tongue followed by buccal mucosa.

Jussawala in 1970[26] studied 2005 cases and found that the high risk sites for tobacco chewers and smokers were vice versa. The chewers had involvement in the

anterior part of oral cavity while the smokers had cancer involving the posterior region i.e. posterior 1/3 rd of tongue, soft palate, oropharynx and tonsils.

Jayant et al in 1977[27] quantified the attributable risk of tobacco chewing in oral cancer. They found that tobacco usuage contributed to 70% of the oral cancers.

Sankaranarayanan et al in 1989[28] studied 187 cases and found that daily frequency of tobacco chewing was the strongest predictor of risk in males, with a relative risk of 15.07 associated with chewing ten or more quids per day. The corresponding relative risk among females was 13.69. In males a relative risk of 3.20 was associated with smoking more than 20 bidis per day, and relative risks of 2.62 and 3.90 were associated with regular use of alcohol and snuff respectively. They concluded that there are four predictors of risk of oral cancer in the chewers/ smokers. They are tobacco daily frequency of tobacco usage, duration of smoking, and alcohol and snuff use (regular versus never). There were also significantly elevated risks associated with occasional indulgence in these four habits.

Znaor et al in 2003[29] studied 1,563 oral, 636 pharyngeal and 566 esophageal male cancer patients and found that tobacco chewing is the strongest risk factor for oral cancer, and smoking for pharyngeal and esophageal cancer. Also there were significant decrease in risks for all 3 cancer sites in subjects who quit smoking.

Schmidt et al in 2004[30] studied 67 OSCC cases and found that 67% of them were smokers. The most common sites of involvement were floor of the mouth and gingiva. This is in contradiction to that of Jussawlla et al and Znaor et al.

Madani et al in 2012[31] gave a detailed description on the various types of tobacco products and their relative risks. This study involved 350 cases. The different indigenous forms of tobacco that is available today include chewable tobacco, paan / pan masala, gutkha, mishiri and beedi. Paan is natural crude areca nut wrapped in betal leaf, with lime, saffron, cinnamon and cloves. This product is now available in immediately consumable forms in sachet. With tobacco, it is gutkha ns without tobacco, it is called paan masala. Supari is a packet of natural crude areca nut without any additives. Mishiri is a chewable tobacco, like a tooth cleaner. The risk was highest for gutkha (7.3), followed by chewable tobacco (5.3), beedi (4.1), supari (4.0) and mishiri(2.2).

C. STUDIES ON BCL2/BAX RATIO IN OTHER MALIGNANCIES

Brambilla et al in 1996[32] studied the apoptosis, bax, bcl2 and p53 in 121 neuroendocrine lung tumours including 16 typical carcinoids , 5 atypical carcinoids, 29 large cell NE carcinomas and 71 small-cell lung carcinomas. Bcl2, bax and p53 were studied by immunohistochemistry. There was inverse correlation between bax and bcl2. Also the bcl2/bax ratio was inversed between the low grade and high grade tumours, there was bax predominance in low grade and bcl2 predominance in high grade tumours. Bc12 overexpression, Bax down-regulation, and Bcl / Bax ratio >1 correlated with lower apoptotic index in tumours with lower survival.

In 2000, Mirjolet et al[33] carried out a study in cell culture of head and neck carcinoma, breast carcinoma and pancreas cancer cell lines to check the sensitivity of 5-

flourouracil, an anticancer drug. The sensitivity of the cells shows a positive correlation with bcl2 and bax ratio, irrespective of p53 gene status.

Scopa in 2001[9] studied the bcl2/bax ratio in 35 rectal carcinoma patients after radiotherapy. Increased bcl2 decreased the prognosis and the opposite was true for bax. Thus the ratio was inversely proportional to the tumour prognosis. The bcl-2/bax ratio was greater in radiresistant group when compared with radiosensitive group and was correlated with poor responsiveness to radiotherapy.

They concluded that the bcl2 / bax ratio can be used as an important molecular marker in predicting the tumour prognosis in rectal carcinoma cases.

In the work by **Saxenna in 2004**[12]**,** a positive correlation between the prognosis and low Bcl-2/Bax ratio similar to the rectal carcinoma was established. They have identified that both Bcl-2/Bax ratio and Mcl-1 are important cell-survival regulators in CLL with pathogenic and clinical significance. A large majority of patients with either low/Bcl-2 Bax ratio or low Mcl-1 or a combination of these findings are likely to exhibit some degree of response to conventional treatment.

In a study by **Matsumoto in 2004**[11]**,** in a retrospective study of advanced bladder cancer cases which were treated by radiation combined with cisplatin therapy immunohistochemistry of bax and bcl2 was done. The bax / bcl2 ratio showed positive correlation with the response to treatment.

In **2012 Kitada et al**[34] studied the expression of Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, and BAD, BAG-1 and Caspase-3 in 58 peripheral blood samples of untreated CLL

patients by immunoblotting technique. Higher levels of Bcl-2 and a high Bcl-2: Bax ratio were correlated with high numbers of white blood cells.

D. STUDIES ON BCL2 / BAX RATIO IN OSCC:

In 1996, Jordon et al[14] studied the expression of bcl2 and bax in 30 OSCC cases and found that 18/ 30 cases (60%) were positive for bcl2 and 19 / 30 cases (63%) were positive for bax. In this study, the pattern of expression of bcl-2 and bax was inversely related to the grade of the tumor with intense immunostaining for bcl-2 and bax in poorly differentiated and well-differentiated SCC respectively. Bcl2 was expressed more in poorly differentiated tumours whereas bax was high among the well differentiated cases. Furthermore, there was strong bcl-2 overexpression in dysplastic areas adjacent to the invasive tumours. The authors believed, based on the expression patterns, that alteration in bcl-2 and bax is likely to play a role in the development of SCC, especially during the early stages of epithelial carcinogenesis.

Staibano S et al in 1998[35] investigated the prognostic role of Cyclin-D1, bcl-2, bcl-1, bax (protein X bcl-2-associated, PCNA and DNA-ploidy proteins in a series of 25 SCC of the oral cavity. In 25 cases 20 cases were with a high/moderate degree of differentiation and five cases were with a low degree of differentiation. In this study they found that expression of Bcl-2 and bax absent in 76% and 60% respectively and present in 24% and 40 % respectively. Bcl-2 showed a weak expression in almost all the cases of SCC of this study, did not show a significant association with any of the other

parameters, such as cellular differentiation, age, sex, site, follow-up (length of time since surgery), disease-free interval and history of smoking, examined. The authors suggested that overexpression of bax seems to constitute an additional indicator of a poor prognosis.

Ito T et al 1999[36] investigated the expression of apoptosis-related factors p53, Bax and Bcl-2 by an immunohisto-chemical approach using an apoptosis index employing an ApopTag kit and also the association between the immunohistochemical results and the clinicopathological data in 57 patients with oral and oropharyngeal SCC. There were six cases of stage I (11%), 11 cases of stage II (19%), 13 cases of stage III (23%), and 27 cases of stage IV (47%) SCC as classified by the general rules for clinical and pathological studies (TNM classification) on head and neck cancer. Of 57 cases 22 (38.6%) were positive for Bax expression. The positive rate of Bax expression was higher at T1/T2 and clinical stage I/ II than at the more advanced stage (T3/T4 and stageIII/IV). Also, the positive rate of Bax expression was higher in the patients without than with lymph node metastasis. The disease-free survival of 5 years was higher in the Bax-positive than the Bax-negative group, but the difference was not significant. There were no associations between Bcl-2 expression and tumor progression, metastasis, recurrence and death due to the disease. Moreover, Bcl-2 expression did not relate to prognosis. Apoptotic index (AI) in Bax-positive tumors was higher than in Bax-negative tumors, but not significant. AI was lower in Bcl-2-positive than Bcl-2-negative cases, but the difference was not significant. They suggested that the spontaneous apoptosis and apoptosis-related protein (p53, Bcl-2, Bax) are not important prognostic factors in

patients with oral and oropharyngeal SCC. However, the changes of apoptosis-related protein expression over primary therapy may contribute to the prediction of prognosis.

In 1999, Loro et al[16] assessed expression of bcl-2 and bax in conjunction with histological grading in oral SCC. They observed a cytoplasmic staining pattern for bcl-2 within the thickness of normal epithelium with intense staining in the basal compartment, but negligible or loss of expression in the basal compartment of well-differentiated SCC. In contrast, bax expression was more intense in the central than in the basal part of the epithelium with higher proportion of bax positive cells in well- and moderately differentiated SCC compared to poorly differentiated SCC. They conclude that, in OSCC, compared with normal tissue, there is a decreased bcl-2 expression, a lowered bcl-2/bax ratio. The expression of bax correlates with histological tumor grading in OSCC.

Homma et al 1999[37] studied whether there is correlation between the treatment outcome and proliferative and apoptotic markers i.e bcl2, bax, p53 and MIB-1 in 111 H&N SCC patients who were concurrently treated with chemoradiation. They found significant correlation between bcl2 and local regional control and also between MIB-1 and overall survival. This finding is controversial with the other studies, where the overexpression of bcl2 is correlated with poor prognosis. Also, no association was found between bax and prognosis. This study also assesses the clinical factors that can predict survival. And it was found that nodal involvement and histological differentiation are associated with poor treatment outcome.

In 1999, Xie et al[18] studied the prognostic values of apoptosis, bax, bcl2 and p53 in OSCC of tongue. This study reveals that low apoptosis index (AI) scores and low bax

expression correlated significantly with poor prognosis, a low Bcl-2 expression was associated with a favorable clinical outcome. Patients with a high bcl-2/bax expression ratio had a significantly poorer prognosis than those with a low ratio. Bax expression, the bcl-2/bax expression ratio, and the TNM classifications were observed as significant independent prognostic variables. However the bcl-2/bax expression ratio was concluded as the strongest independent prognostic parameter.

In 2000, Chen et al[17] evaluated the bcl2 and bax expression in OSCC and the bax mRNA and bcl2 mRNA by reverse transcriptase polymerase chain reaction. It was observed that the expression level of bcl-2 mRNA or bax mRNA was not consistent with their protein level in some cases. Higher expression of bcl-2 mRNA and stronger immunostaining of bcl-2 protein were found in oral OSCC than in the adjacent histologically normal OE. These findings were more prominent in poorly differentiated cases. No significant differences in bax mRNA and protein were observed between OSCC and the adjacent histologically normal OE. However, poorly differentiated carcinomas showed very weak immunostaining for bax. The ratio of bcl-2/bax mRNA was higher in OSCC than in the adjacent histologically normal OE, and higher ratios were seen in most of poorly differentiated OSCC. This study supplies indirect evidence of post-transcriptional control of bcl-2 and bax expression, and suggests that dysregulated expression of bcl-2 and bax may be related to the differentiation of OSCC.

Loro et al in 2000[38]evaluated the expression of bcl2, bax, ki 67 and apoptosis in 13 OSCC cases from sudan with toombak habit (snuff dipping) and 6 non-users from the Sudan and Norway by immunohistochemistry. Apoptosis was evaluated by the TUNEL method. They found a higher apoptotic rate and a higher bax expression in OSCC from

Norway compared with those from the Sudan ($p < 0.05$) irrespective of toombak use. No significant differences were detected in apoptosis, bax, bcl-2 and Ki-67 in OSCC from the Sudan in relation to toombak use. They concluded that in OSCC, apoptosis was associated with bax expression and was unaffected by p53 gene status or toombak use in OSCC from the Sudan.

Teni et al in 2002[39] assessed immunohistochemical expression of bcl2, bax and p53 in 63 oral squamous cell carcinoma patients among Indian population, to study the involvement of these apoptosis regulating proteins in OSCC in a population with habit of tobacco chewing. All of them had a history of tobacco chewing habit for a minimum of atleast 10 years. Of the 63 cases, 17 were well differentiated, 29 were moderately differentiated and 17 cases were poorly differentiated OSCC. They also included 11 premalignant lesions including oral leukoplakia and oral submucous fibrosis cases in their study. They observed similar expression of both bcl2 and bax in the central cells of tumor islands and in the superficial epithelial layers of oral lesions. 56% of oral cancers and 16% of oral leukoplakia were positive for bcl2, bax was positive in 43% of oral cancers and 55% of oral lesions, p53 was positive in 54% of oral cancers and 43% of oral lesions. 30% of oral cancers demonstrated p53+bcl-2+ pattern and 14% of oral cancers were positive for all the 3 proteins. But none of the oral lesions showed overexpression of p53 and bcl-2 or all the three genes. Thus the bcl2, bax and p53 overexpression were concluded to have a critical role in the development of Indian OSCC by allowing apoptosis and also over expression in premalignant conditions suggest possible role of early carcinogenesis.

The other findings in this study were, bcl2 expression significantly correlated with nodal status and therefore poor prognosis, and in contrast to all other studies of bcl2 and bax, the authors observed overexpression of both bcl2 and bax was more in moderate differentiated OSCC.

In 2003, Sulkowska et al[40] studied the correlation between bcl2 expression and clinicopathologic features in 129 OSCC cases. The study was conducted on 129 patients treated surgically for OSCC. Positive immunoreactivity for Bcl-2 was present as a coarse or finally granular cytoplasmatic staining, irregularly distributed around nuclei. Marked Bcl-2 expression was observed in the peripheral cells of squamous cell carcinoma focuses, diminishing toward their centers. Strong positive immunohistochemical staining was also observed in poorly differentiated squamous cell cancers and most cancer microfocuses at the tumor invasive margins.

The statistically significant relationships were observed between oral squamous cell cancer Bcl-2 expression and higher tumor grading ($p<0.005$), higher tumor mitotic index ($p<0.005$), higher index of atypical mitoses ($p<0.001$) as well as microfocal pattern of tumor invasive margin ($p<0.001$). The results suggest that positive Bcl-2 expression may be a valuable factor supplementing the established unfavorable histopathological features of OSCC.

In 2005, Assis et al[41] made experimental trials in rats by exposing them to cigarette smoke and studied the changes in bcl2, bax, and PCNA between the experimental group and negative controls. the labeling index for bcl-2 and bax showed an increase 75 days after cigarette exposure. PCNA-labeling index did not show remarkable

changes between groups. An overexpression of bcl-2 was detected throughout all layers of the epithelium, whereas bax did not show significant differences.

Loro et al in 2005[42] in continuation to their work in 1999, they **s**tudied whether the underexpression of bcl2 in epithelial dysplasia and OSCC and that of bax in poorly differentiated OSCC is due to any mutations in bcl2 and bax, to correlate the expression pattern and to ascertain the mutational spectrum of these genes in epithelial dysplasia and oral SCC. This was done by immunohistochemical analysis of bcl2 and bax in 22 cases of oral epithelial dysplasia and 28 cases of OSCC and genomic extracton was done by in situ hybridisation from the paraffin blocks, and is then amplified. Tonsillar tissue is used as the positive control. Bcl2 was positive in the mantle zone and the bax was positive in the follicular centres of tonsillar tissue. They found that the expression of bax was widely noted in tumor cells of well-differentiated but not in poorly differentiated SCC compared to the low level of expression of bcl-2 in both histological grades of SCC. The authors concluded that loss of bcl-2 in basal cells of epithelial dysplasia and SCC as well as loss of bax in poorly differentiated SCC are not associated with mutations in the coding regions of these genes.

De Vincent et al in 2006[43] studied the expression pattern of bcl2 and bax in 35 tongue cancr cases and also to correlate it with the clinicopathologic features and prognosis. Bax was detected in 37.1% (13 of 35 cases). Bcl-2 was only immunoexpressed in 8.6% (3 of 35 cases). Both bcl2 and bax showed granular cytoplasmic positivity that was strong in the periphery of tumour islands which decreased towards centre. Carcinoma breast cells are used as control for bax and tonsil was used for bcl2, where it stained only the mantle zone. Bax correlated with histologic grading and

bcl2 with nodal status and poor prognosis. There was no correlation between bax or bcl2 and age, sex, and T- size. It was concluded that bcl2 can be used as a predictor of patient's survival.

Jane et al in 2006[44] analyzed the expression of surviving, bcl2, bax and p53 in 38 cases of OSCC and 17 leukoplakia cases among Indian population. Of the 38 OSCC cases, 13 were well differentiated, 14 were moderately differentiated and 11 were poorly differentiated. Breast carcinoma was used as positive control. The immunohistochemical analysis was based on the percentage cells stained. Bcl-2 staining cytoplasmic and in leukoplakia it was found in the keratin and prickle cell layer in weak to moderate intensities. 92.3% of well differentiated caese (12 out of 13 cases) showed weak to moderate Bcl-2 expression. 57.14% of moderately differentiated cases showed moderate Bcl-2 expression and 100% of poorly differentiated cases showed strong Bcl-2 expression. Bax was positive in the prickle cell layer and the basal layer of leukoplakia. 84.62% of well differentiated OSCC, 90.91% of moderately differentiated OSCC and all of the poorly differentiated OSCC were positive for bax PDSCC. Thus both bax and bcl2 increased with decrease in degree of histological differentiation of OSCC. Similar results were found for surviving and p53 protein.

In 2007, Kummoona et al[45] studied the expression pattern of bcl2 in 24 cases of oral cancer and correlated it to the clinicopathological features like patient's age, tumour size, lymph node metastasis. But none of the correlation was statistically significant.

In 2008, Kato et al[46] suggested that bax is a significant predictor of prognosis and is correlated with bcl2 and bax. Bax expression were significant and independent

variables. Bax expression was found to be the strongest independent prognostic parameter. Patients with negative Bcl-2 expression and positive Bax expression had a significantly better prognosis ($P < 0.005$).

Zhang et al in 2009[47], studied 110 OSCC cases and analysed the bcl2 / bax ratio and correlated with the parameters like histological grading, clinical staging and prognosis. Positive correlation was found only with prognosis. It was concluded that the treatment planning for the patients with bcl2 / bax >1, should be more intensive.

Camicassa et al in 2009[47] analyzed 53 OSCC cases clinically for TNM staging, histologically for lymphocytic infiltrate, pattern of invasion, perineural invasion, degree of differentiation and also immunohistochemically for bcl2, bax and bcl-x. These factors were correlated with prognosis. All of these factors were predictive of survival outcome. Tongue cases had low survival rate than other sites. Digital image analysis was used to assess the immunohistochemical staining of bcl2, bax and bcl-x. Bcl2 and bcl-x were correlated with decreased survival and bax was associated with better survival.

De souse et al in 2009[49] made a comparative analysis of bcl2, bax, p53 and PCNA in OSCC and Lichen planus. In the study, of the 24 OSCC cases bax was positive in 66.7% were positive for bax and 16.67% were positive for bcl2. There was correlation between bax and PCNA and between bcl2 and p53 only in lichen planus and no such correlation was observed in OSCC.

Camillo et al in 2010[15] carried out an extensive study involving multiple proteins of the bcl2 family that either inhibit (Bcl-2, Bcl-x, Bcl-xL, Bcl-2-related protein A1, BAG-1) or promote (Bak, Bax, Bim ⁄ Bod, Bim-Long, Bad, Bid, PUMA)apoptosis

and also to establish any correlations between the expression of these proteins and clinicopathological features of OSCC like tumour site, clinical stage, nodal involvement, vascular invasion, perineural infiltration, histological grade and recurrence of OSCC. Bax was associated with absence of vascular invasion and with tumours occurring in the oral tongue. Bcl2 was not associated with any of the clinicopathological factors studied.

Ranganathan et al in 2011[50] attempted to compare the proliferative (Ki67) and apoptotic markers (bcl2, bax and p53) between 50 cases of OSMF and 10 cases of OSCC. 10 normal oral epithelial tissue samples. The bcl2 was positive only in one case of OSMF and one case of OSCC and in none of the normal oral epithelium. Bax showed cytoplasmic positivity in 8 normal oral epithelium, 38 OSMF and all cases OSCC. The bax staining was seen in the basal and suprabasal layers both in the normal samples and OSCC cases.

Zhao et in 2011[51] studied the relationship between the apoptosis and the bcl2, bax proteins in 15 cases of maxillofacial squamous cell carcinoma, after exposing them to thermochemotherapy (microwave induced hyperthermia + bleomycin injection). Out of these 15 cases, 9 patients had lip cancer and 6 of them had facial skin cancer. Histologically, tumors were graded as poorly differentiated (3 cases), moderately differentiated (8 cases), and well differentiated (4 cases). Bcl2 and bax expression was studied by immunohistochemistry before and after thermochemotherapy. The results showed that bcl2 was downregulated and apoptosis, bax were upregulated after the procedure. The authors conclude stating that the therapeutic procedures for SCC act by upregulating the pro- apoptotic proteins and by down regulating bcl2.

Jham et in 2012[52] studied the expression of MK (midkine), Ki-67, PCNA, p53, bcl-2, Bax, and CD31 in 28 OSCC cases. The expression of bcl-2 was increased in MK-positive tumors. This was in contrast to the expression bax, which was unaltered in MK-positive cells. Therefore, the authors conclude saying that the MK participates in tumorigenesis via increased expression of anti-apoptic proteins, leading to a decrease in cell death.

Bose et al in 2012[53] studied the association between apoptosis-regulating proteins (bcl2, bax and bcl-Xl) and clinical outcomes in OSCC using the quantitative fluorescence immunohistochemistry (IHC) based AQUAnalysis technique. This was done both in normal oral epithelium and in 69 OSCC cases. They observed a nuclear positivity of bax in the basal layers of normal epithelium and cytoplasmic overexpression in the OSCC samples.

E. STUDIES ON IHC ANALYSIS OF BCL2 & BAX

In **Nakawaga et al in 1994**[54]**,** the positive cells were scored in several randomly selected fields at X400 magnification. The percentage of positive cells was classified as follows: (+++: strongly positive) more than 50% of the total cells were stained against bcl-2 antibody in the cytoplasm; (++: moderately positive) 25-50% of the total cells were stained; (+: weakly positive) 5-25% of the total cells were stained; (-: negative) less than 5% of the total cells were stained.

Xie et al in 1999[18]**, studied t**he immunopositivity of bcl2 and bax by adding the % of Bcl2 and Bax positive cells and adding it with the intensity of staining. The score for % of positive cells are: 1 to 30 % of positive cells – 1 31 to 70% of positive cells – 2 and 71

to 100% of positive cells – 3. Intensity of staining is scored as Strongly positice- 3, moderately positive – 2, weakly positive – 1.

MATERIALS AND METHODS:

SOURCE:

Out-patient department,

Department of Oral Pathology,

Tamil Nadu Government Dental College & Hospital,

Chennai- 600 003.

SUBJECTS

Histologically confirmed oral squamous cell carcinoma patients, irrespective of age and gender.

SAMPLE SIZE

All cases of oral squamous cell carcinoma patients who will be reporting to the department to receive their biopsy report (January 2012 to June 2012), will be prospectively included in this study. The expected sample size is 60.

INCLUSION CRITERIA

1. Patients with histologically proven cases of oral squamous cell carcinoma who had signed the informed consent.
2. OSCC cases of any stage of TNM classification.
3. OSCC Cases of any histological grading.
4. Patients with or without habit of smoking or tobacco chewing of any form.
5. Patients with or without history of trauma.

EXCLUSION CRITERIA

1. Cases without unequivocal evidence of squamous cell carcinoma.
2. Patients with more than one etiological factor.

METHODOLOGY:

- History of Tobacco chewing/ smoking/ trauma is recorded from the patient.
- Patient is clinically examined and TNM staging of the tumour (by Pierre Denoix between 1943 and 1952) is recorded.
- H & E sections (4µ thickness), made from paraffin blocks of the patient are examined and histological grading is done.
- Immunohistochemical analysis of Bcl2, Bax are made in the paraffin sections (4µ thickness) for all cases, using the avidin-biotin peroxidase technique (by Hsu et al 1981)[6], with relevant positive and negative controls.

❖ **IMMUNOHISTOCHEMICAL PROCEDURE:**

- 4 micron thickness are made on amino propyl triethoxy silane (APES) slides.
- The slides are deparafinized and heat fixed at 55 to 60 degrees centigrade overnight.
- They are then treated with following washes

    1. Xylene: 2 x 3 minutes
    2. Xylene 1:1 with 100% ethanol: 3 minutes
    3. 100% ethanol: 2 x 3 minutes
    4. 95% ethanol: 3 minutes
    5. 70 % ethanol: 3 minutes
    6. 50 % ethanol: 3 minutes
    7. Running cold tap water to rinse

- Antigen retrieval: Place the slide rack in Tris-EDTA buffer (9 pH) in a pressure cooker for about ten minutes (3 whistles)
- The pressure cooker is then bench cooled for 20 minutes.
- Slide rack is then taken out, and washed in running water and distilled water.
- Slides are then placed in a humidified chamber.
- Sections are then treated with 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity.
- Slides are washed in tris buffer (pH 7.6)
- Sections are then treated with power block

- Treat with primary antibody (anti bax / anti bcl2) for 1 hour.
- Tris buffer wash
- Treat with secondary antibody for 30 minutes.
- Tris buffer wash.
- Stain with DAB (3-diaminobenzidine) chromogen.
- Wash with distilled water.
- Counterstain with hematoxylin.

❖ SCORING OF BCL2 AND BAX EXPRESSION:

The % of Bcl2 and Bax positive cells will be calculated semiquantitatively, irrespective of the intensity of staining by a applying the modifiesd semiquantitative method followed by Xie et al in 1999, [21]

1 to 30 % of positive cells – 1

31 to 70% of positive cells – 2

71 to 100% of positive cells – 3

Scoring is done in atleast 4 or 5 high power fields per slide and the average score is noted for that slide. The ratio is then calculated.

❖ CLINICOPATHOLOGIC EVALUATION:

The Bcl2/Bax ratio will be interpreted in the context of the following clinicopathological parameters:

1. Age

2. Sex
3. Site
4. Presence or absence of Trauma / Smoking / Tobacco history
5. TNM Stage
6. Histological grade.

STATISTICAL ANALYSIS:

The collected data was analysed with SPSS 16.0 version. To describe about the data descriptive statistics mean, S.D were used. To find the significance difference between the bivariate samples independent t-test test was used. For the multivariate analysis the analysis of variance (ANOVA) with Post-hoc test Tukey's HSD was used to find the significance difference between the inter group comparison . In all the above statistical tools the probability value P=.05 is considered as significant level.

Fig 1. Primary anti bax & anti bcl2 antibodies

Fig 2. Secondary poly-hrp antibody kit

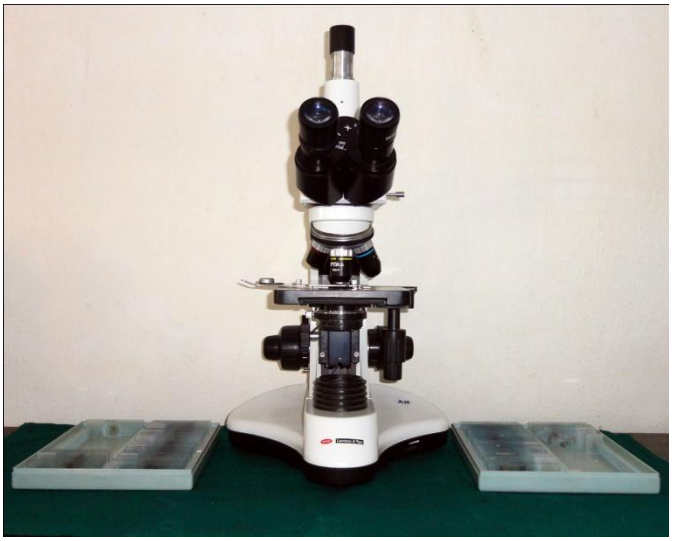

Fig 3. Light microscope used for analysis of

bcl2 & bax immunostained slides

GENERAL DISTRIBUTION

The gender ratio was 1.87 with the distribution showing a male predominance with 65% males cases (39/60 cases) and 35% female cases (21/60 cases). The age of the patients ranged from 26 to 86 years. Patients were categorized into three age groups for comparison purpose. They are 25 to 45 years, 46 to 65 years and 66 to 86 years. 55% of the patients belonged to the 46-65 years age group, 30% belonged to 25-56 years age group and 15% of the patients belonged to 65-86 years age group. The buccal mucosa was the most common site and formed 30% (18/60 cases), this was followed by alveolar mucosa (26.66% or 16/60 cases), retro molar zone (21.67% or 13/60 cases) and tongue (21.67% or 13/60 cases).

All of the patients had a history of atleast one habit, and the gutkha / maawa chewing habit was the most common habit among the cases (35% i.e 21/60 cases), followed by betal quid chewers (30% or 18/60 cases), smokers (16.67% or 10/60 cases), snuff users (10% or 6/60 cases) and 8.3% of patients had history of trauma (5/60 cases). All of them who had history of trauma belonged to the tongue OSCC cases.

Among the 60 cases studied, 57 cases (95%) were of well differentiated OSCC, and 7 cases are of (11.67%) were of moderately differentiated type. Not a single case of poorly differentiated OSCC was recorded during the period.

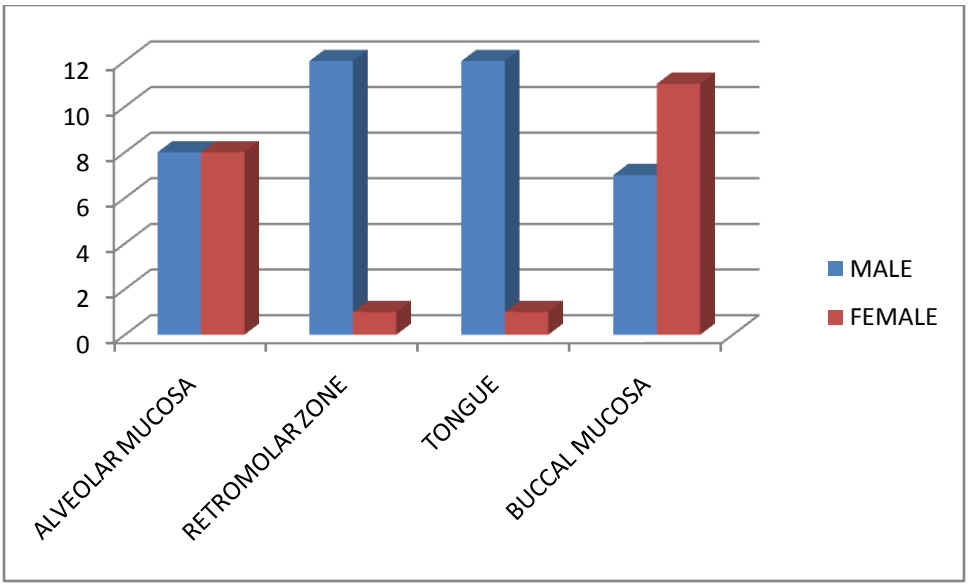

Fig.4: Gender distribution

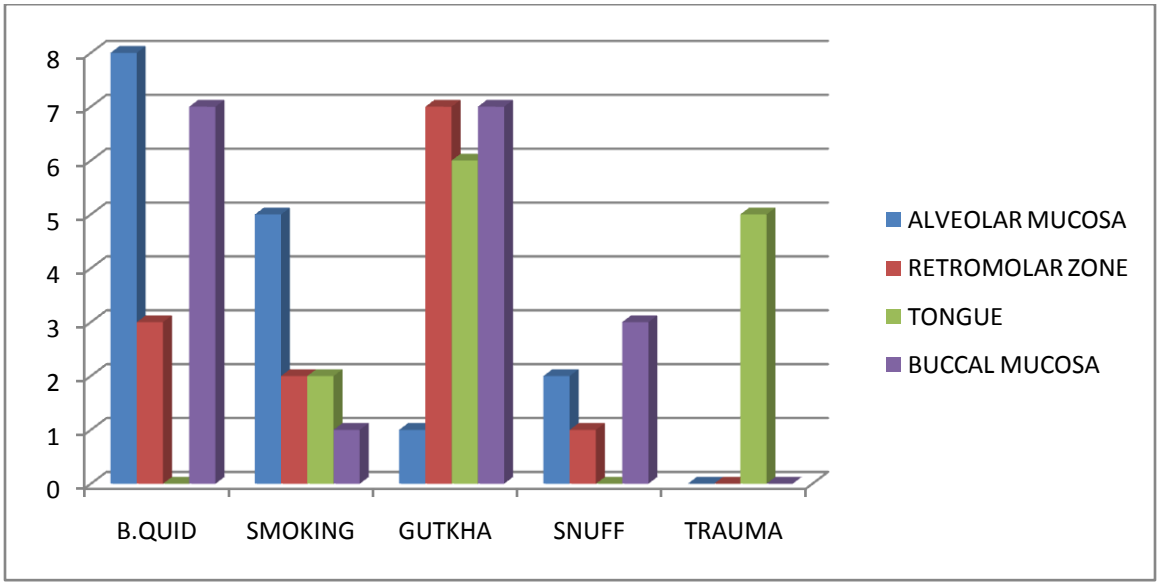

Fig.5: Distribution of habits

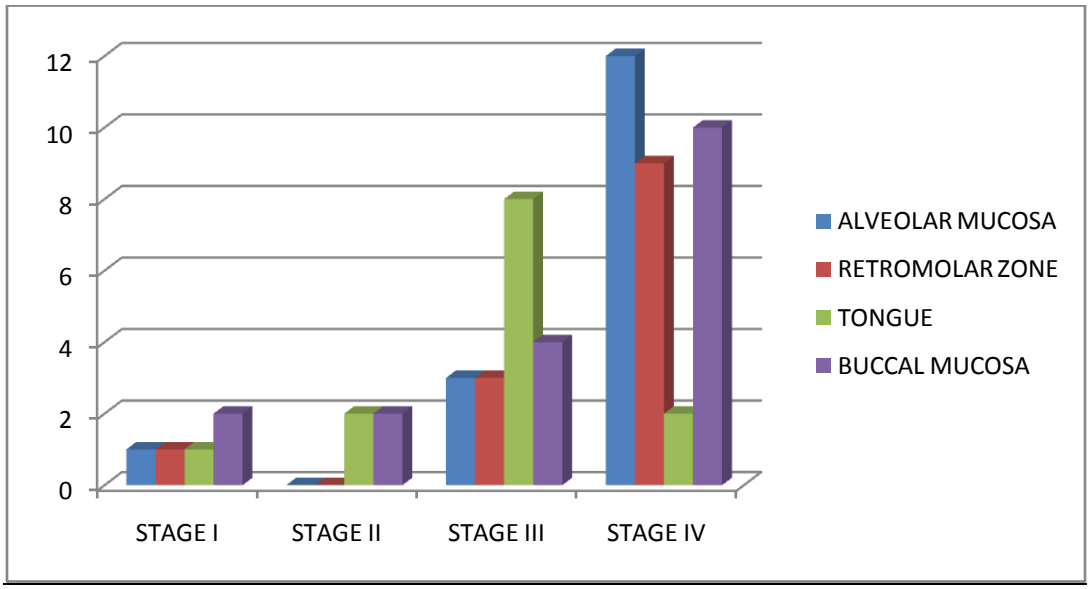

Fig.6: Distribution of diseases stage

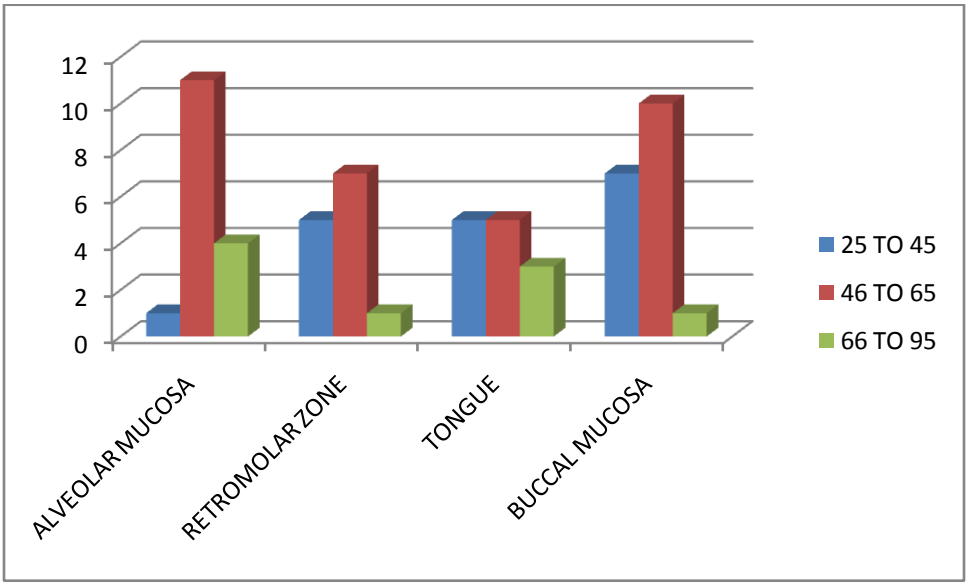

Fig.7: Age distribution

IMMUNOHISTOCHEMISTRY RESULTS

CONTROL TISSUE:

- **BCL2:**

  Diffuse, strong, cytoplasmic positivity in the mantle zone and interfollicular area of tonsil tissue. (Fig.8)

- **BAX:**

  Diffuse, strong, cytoplasmic positivity in the tumour epithelial islands of carcinoma breast. (Fig.9)

NORMAL EPITHELIUM:

- BCL2:

  Moderate to strongly cytoplasmic positivity seen in the basal layers. (Fig.10)

- BAX:

  Moderate to strong cytoplasmic positivity seen in basal and suprabasal layers of the epithelium. (Fig.11)

OSCC SAMPLES:

- BCL2:

  Diffuse, weak to moderate cytoplasmic positivity seen in the islands and dysplastic epithelium. Majority of the samples were weakly positive.(Fig.14, 15, 16)

- BAX:

Diffuse, moderate to strong cytoplasmic positivity, sometimes strong only in the peripheral cells of tumour islands and sometimes stronger throughout the entire thickness of tumour island was seen. (Fig 12, 13, 17)

BCL2 & BAX IN CONTROL TISSUES

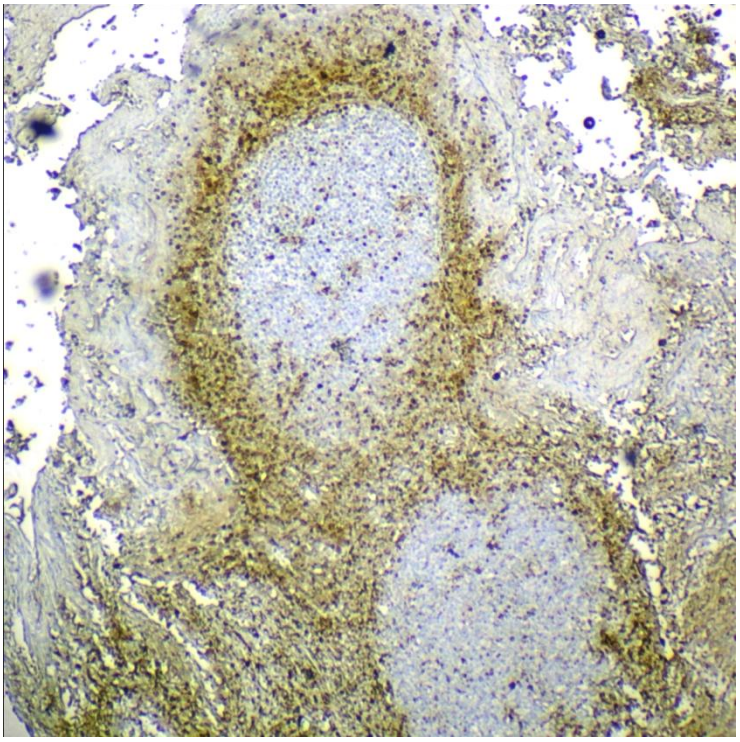

Fig.8: Bcl2 staining in control tissue – Tonsil (mantle zone & interfollicular area)

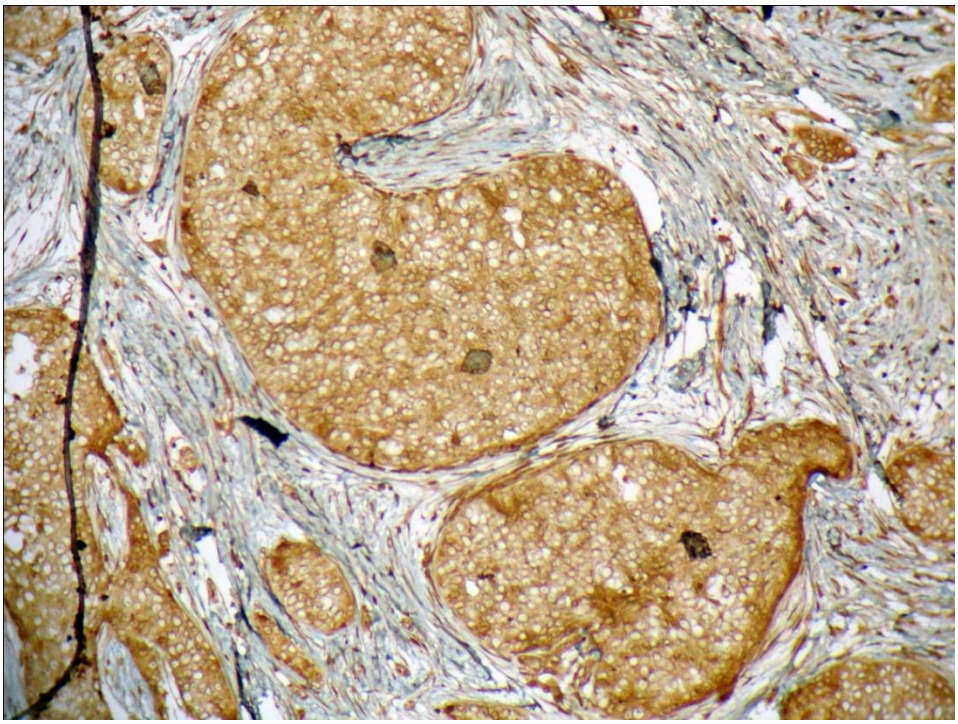

Fig.9: Bax staining in the control tissue – Breast carcinoma

BCL2 & BAX IN NORMAL EPITHELIUM

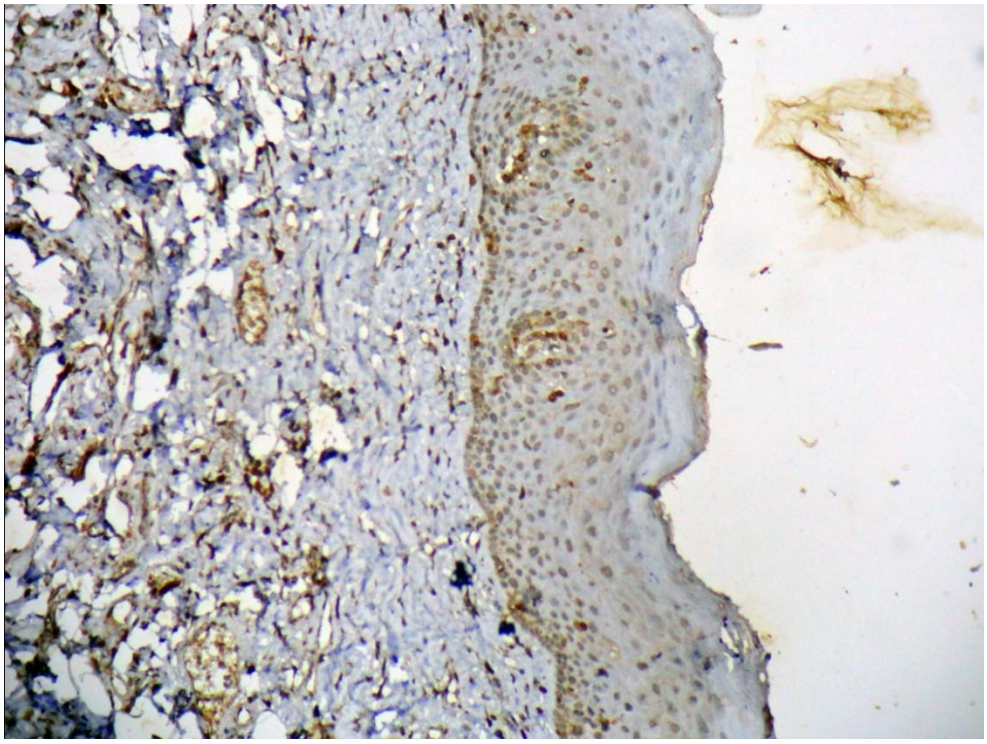

Fig.10: BCL2 In normal oral epithelium – Strong in basal cells.

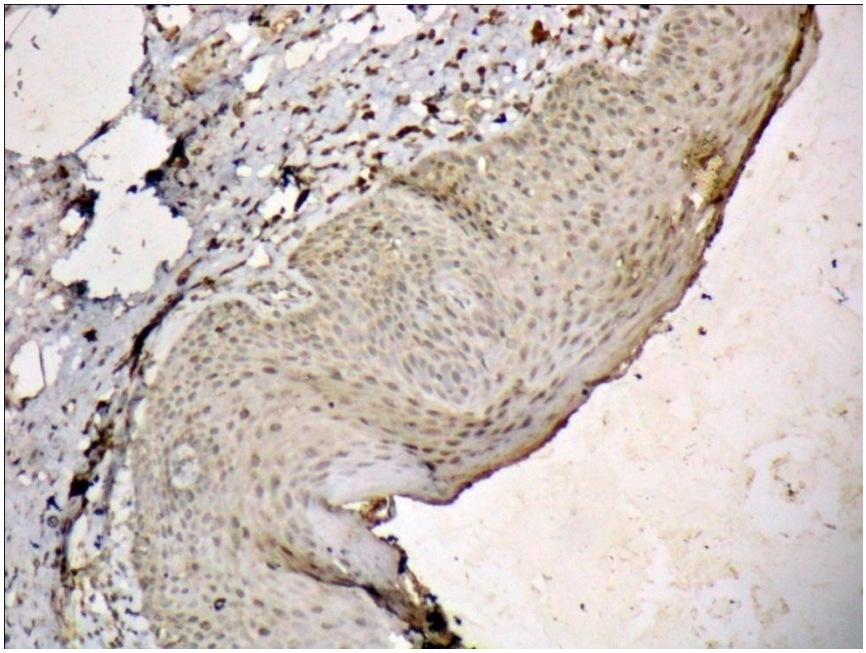

Fig.11: Bax in normal epithelium – stronger throughout the thickness.

BAX IN TEST SLIDES

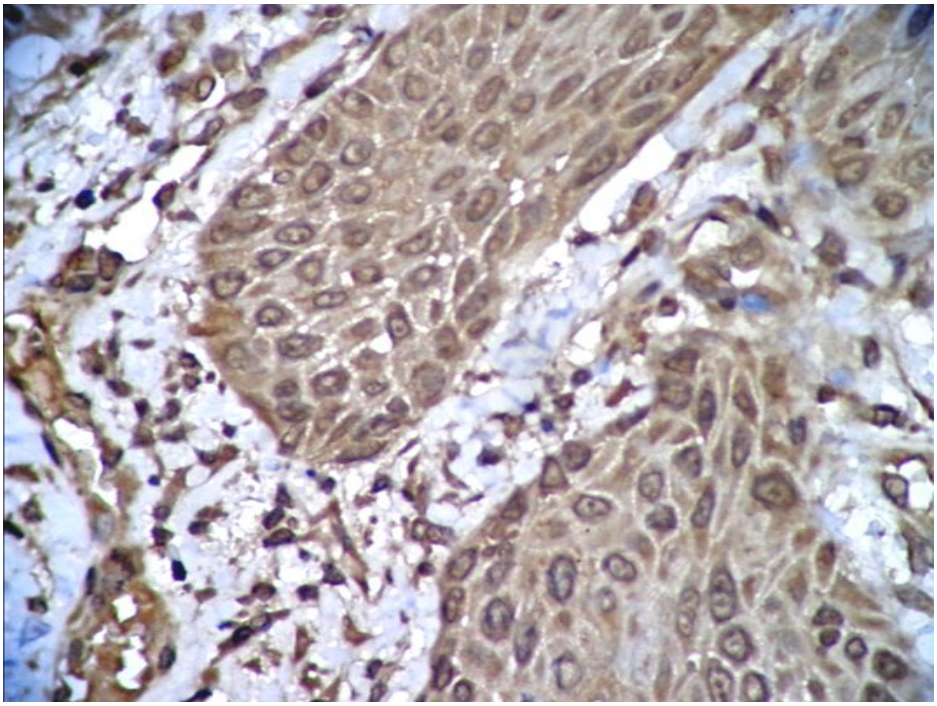

Fig.12: Strong bax positivity in well differentiated tumour in 40X

(score 3/HPF)

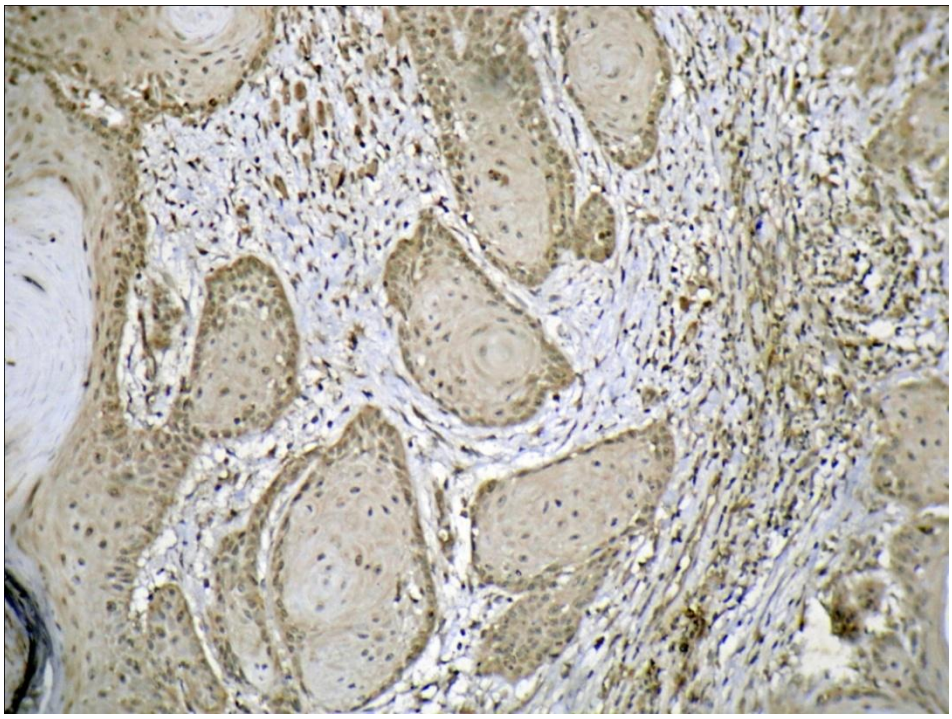

Fig.13: Bax in low power view (few areas can be given a score of 2, others 3/HPF)

BCL2 IN TEST SLIDES

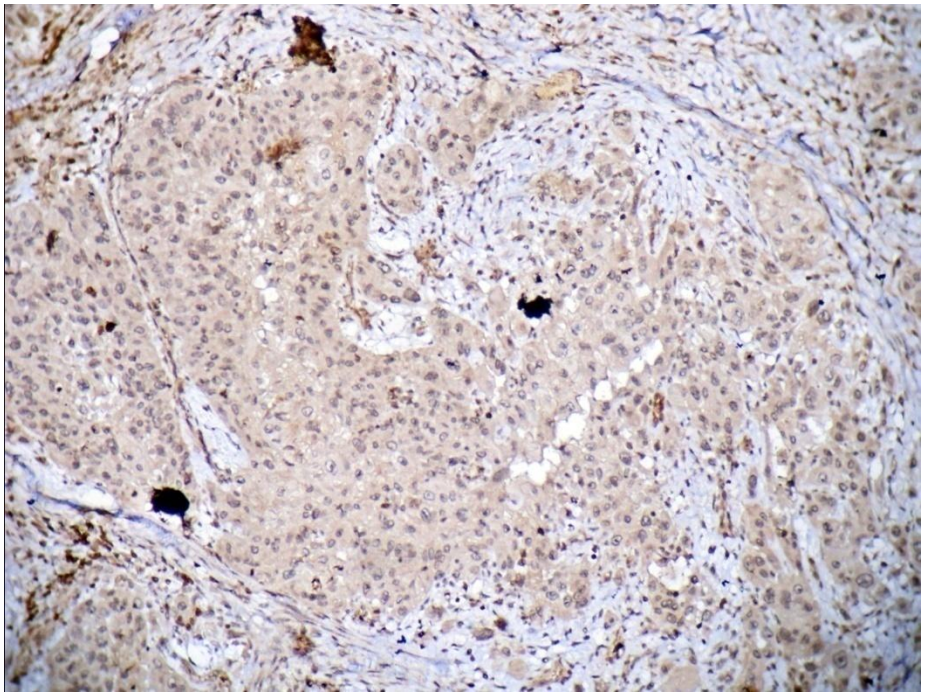

Fig.14: Strong immunopositivity of bcl2 in Moderately differentiated tumous (Score 3/HPF)

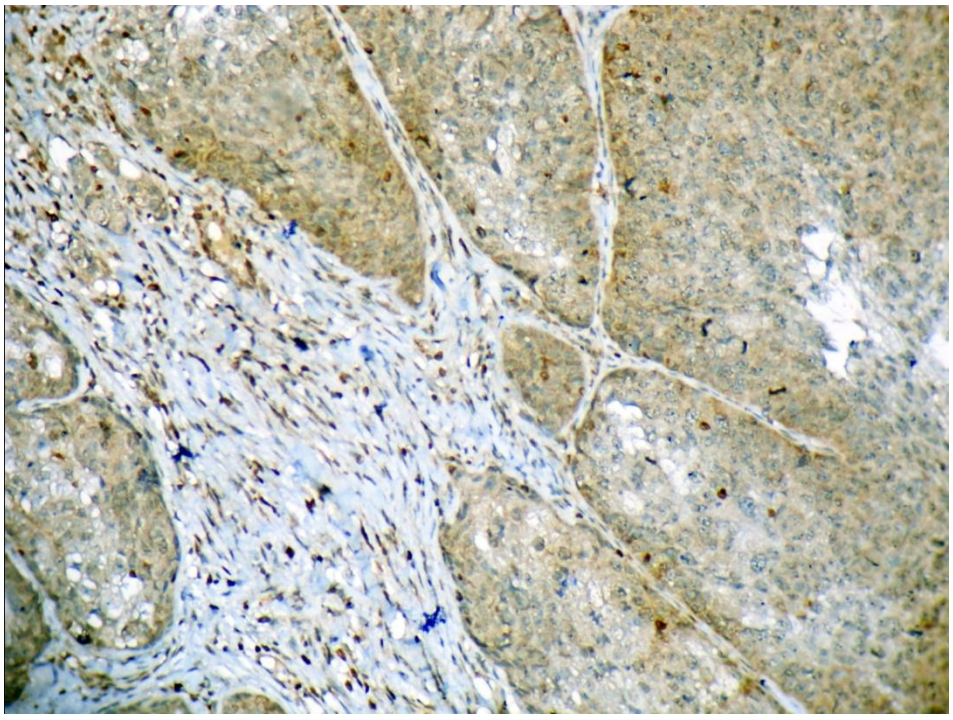

Fig.15: Bcl2 in showing intermixed positive and negative cells (Score 2/HPF)

COMPARISON OF BAX & BCL2 IN A CASE

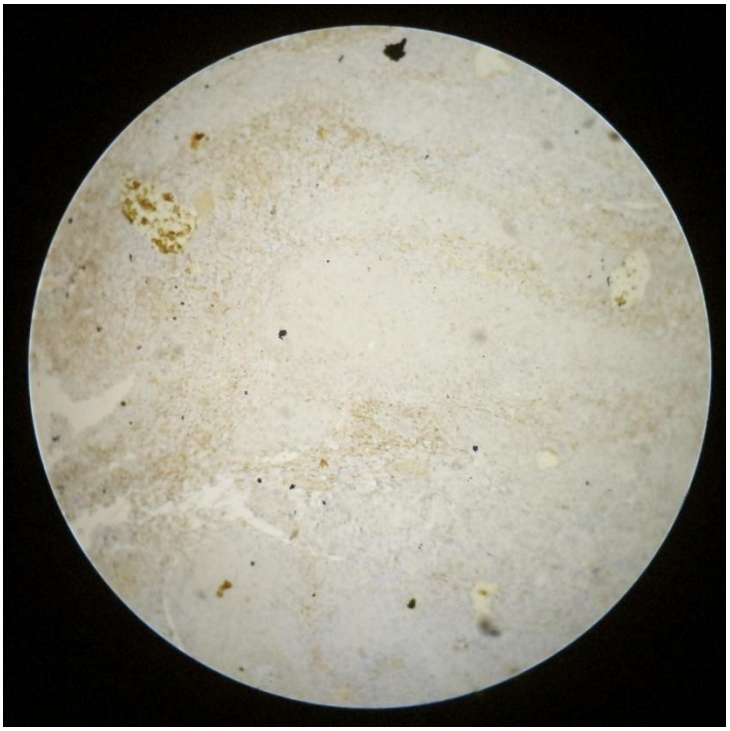

Fig.16: Weakly positive bcl2 (score 1/HPF)

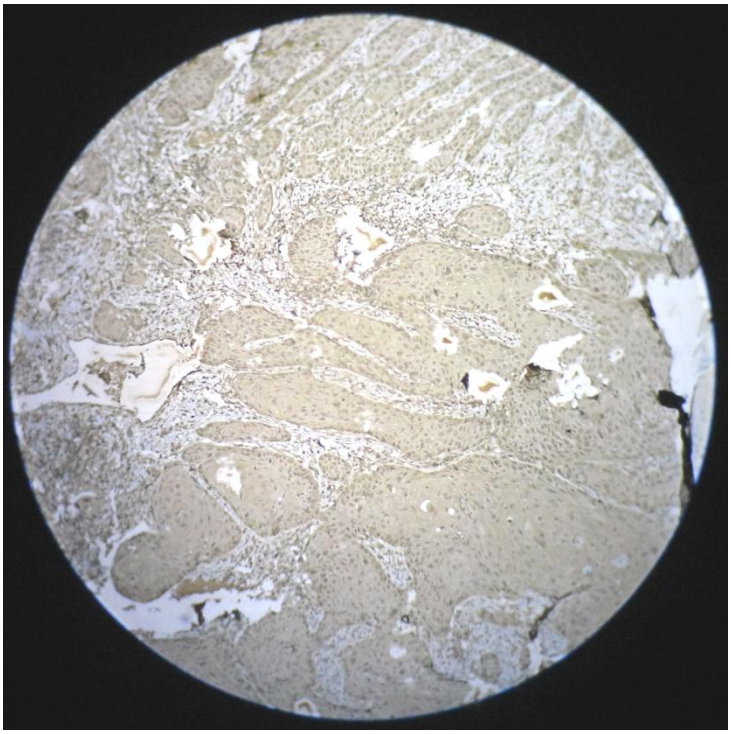

Fig.17: Strongly positive bax in the same focus (score 3/HPF)

ANALYSIS OF BCL2/BAX RATIO (Fig. 18)

The difference between the bcl2/bax was not significant between the OSCC cases that involved buccal mucosa, alveolar mucosa and retromolar zone. But the bcl2/bax was significantly different between the tongue cases and the cases that involved other sites. The subsites were then classified according to the age groups, sex, habit and clinical stage. The bcl2/bax values were tabulated accordingly.

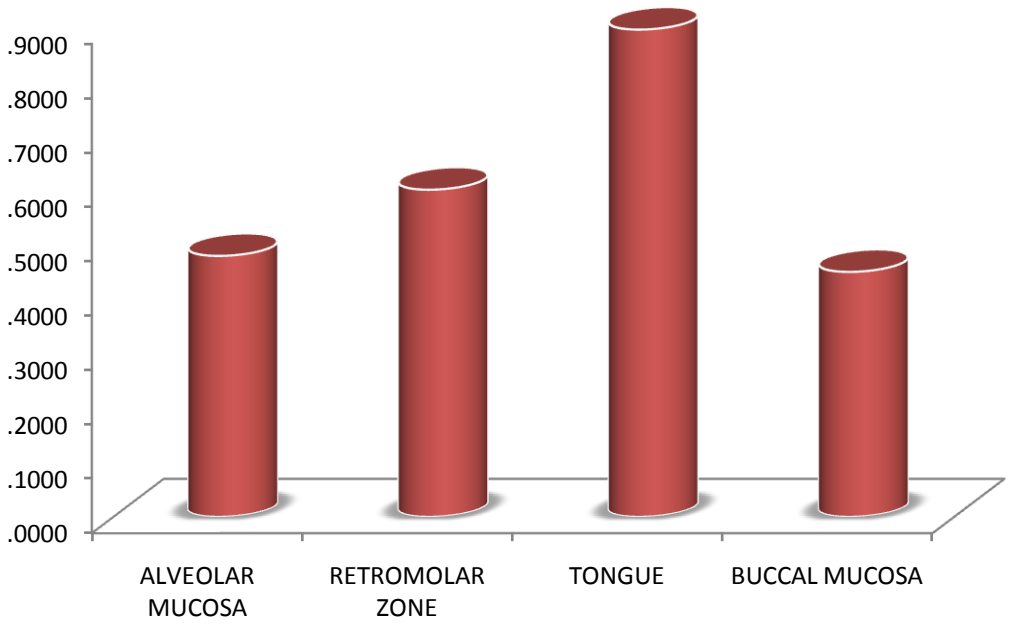

Fig.18: Comparison between the bcl2/bax ratio between sites

ANALYSIS OF BCL2/BAX AMONG THE BUCCAL MUCOSA CASES

(Table 1a,b,c)

Among the buccal mucosa cases, 62.50% were female (10/16 cases) and 37.50% were male (6/16 cases) with a sex ratio of 0.6. This contradicted with the overall sex ratio. The age distribution was however similar to that of the overall picture. The majority of the cases belonged to the 46-65 years group (56.25% or 9/16 cases), with 37.5% in 25-45 years age group (6/16 cases) and 6.25% in the 66-86% age group (1/16 cases). Equal number of cases had betal quid and maawa/gutkha chewing habits (37.5% or 6/16 cases each). This was followed by snuff dipping habit (18.75% or 3/16 cases) and only one case had habit of smoking (6.25%). 50% of cases belonged to the stage IV of TNM classification. 25 % in the stage III, 12.5% each in stage I and II. None of these factors significantly affected the bcl2/bax ratio.

Table 1a: Comparison Of Bcl2/Bax Between The Age Ranges

Age Ranges In Years	Mean	Std. Deviation	P- value	Sig.
25 TO 45	0.4283	0.11839	0.510	NS
46 TO 65	0.4822	0.17570		
66 TO 95	0.3000	.		

Table 1b: Comparison Of Bcl2/Bax Between The Habits				
Habit	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
B.Quid	.3767	.09832	.309	NS
Smoking	.5000	.		
Gutkha /maawa	.4500	.14339		
Snuff	.5833	.23714		

Table 1c: Comparison Of Bcl2/Bax Between The TNM Stages				
TNM Stage	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Stage I	.4750	.10607	.339	NS
Stage II	.2600	.05657		
Stage III	.4800	.13266		
Stage IV	.4775	.16960		

ANALYSIS OF BCL2/BAX AMONG THE ALVEOLAR MUCOSA CASES

(Table 2a,b,c)

Among the alveolar mucosa cases, 53.30% were male (8/15 cases) and 46.70% were female (7/15 cases) with a sex ratio of 0.87. This contradicted with the overall sex ratio. The majority of the cases belonged to the 46-65 years group (73.30% or 11/15 cases) and the remaining 26.7% is in 66-95 years age group (4/15 cases). In this group most of the patients had betal quid habit (53.30% or 8/15 cases) followed by smoking and snuff dipping habit (33.3% or 5/15 cases and 13.40% or 2/15 cases respectively). 73.30% of cases belonged to the stage IV of TNM classification, 20% in the stage III and 6.70% in stage I. None of these factors significantly affected the bcl2/bax ratio.

Table 2a: Comparison Of Bcl2/Bax Between The Age Ranges				
Age Ranges In Years	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
AGE 46 TO 65	.4591	.15469	.403	NS
AGE 66 TO 95	.5375	.15756		

Table 2b: Comparison Of Bcl2/Bax Between The Habits				
Habit	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Betel quid	.4800	.18639	.804	NS
Smoking	.5060	.12482		
Snuff	.4150	.12021		

Table 2c: Comparison Of Bcl2/Bax Between The TNM Stages				
TNM Stage	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Stage I	.6600	.	.446	NS
Stage III	.5100	.14526		
Stage IV	.4555	.15744		

ANALYSIS OF BCL2/BAX AMONG THE RETROMOLAR ZONE CASES

(Table 3a,b,c)

Among the retromolar cases, 91.90% were male (10/11 cases) and 9.10% were female (1/11 cases) with a sex ratio of 0.1. This contradicted with the overall sex ratio. The majority of the cases belonged to the 46-65 years group (54.50% or 6/11 cases) and 45.5% is in 25-45 years age group (5/11 cases). Maximum number of patients had gutka habit (45.5% or 5/11 cases) followed by betal quid habit (27.20% or 3/11 cases) smoking (18.20% or 2/11 cases) and snuff dipping habit (9.10% or 1/11 cases). 72.70% of cases belonged to the stage IV of TNM classification, 18.20% in the stage III and 9.10% in stage I. None of these factors significantly affected the bcl2/bax ratio.

Table 3a: Comparison Of Bcl2/Bax Between The Age Ranges				
Age Ranges In Years	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Age 25 to 45	.5980	.25322	.957	NS
Age 46 to 65	.6050	.16294		

Table 3b: Comparison Of Bcl2/Bax Between The Habits				
Habit	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Betel quid	.7667	.25166	.291	NS
Smoking	.6650	.23335		
Gutkha/ maawa	.4980	.11670		
Snuff	.5000	.		

Table 3c: Comparison Of Bcl2/Bax Between The TNM Stages				
TNM Stage	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Stage I	.3300	.	.247	NS
Stage III	.7450	.12021		
Stage IV	.6000	.19272		

ANALYSIS OF BCL2/BAX AMONG THE TONGUE CASES

(Table 4a,b,c)

Among the tongue cases, 91.90% were male (10/11 cases) and 9.10% were female (1/11 cases) with a sex ratio of 0.1. This contradicted with the overall sex ratio. The majority of the cases belonged to the 25-45 years group (45.60% or56/11 cases) followed by 27.20% is in both 45-65 years and 66-95 years age group (3/11 cases in each group). 45.50% patients had gutka/maawa habit (45.5% or 5/11 cases) and 45.50% had trauma followed by smoking habit (9% or 1/11 cases). 72.70% of cases belonged to the stage III of TNM classification, followed by 18.20% in the stage II and 9.10% in stage I. In this site habits or the risk factors affects the bcl2/bax expression as it shows the

significant difference in bcl2/bax ratio between trauma group and smoking and gutkha group(Fig.6).

Table 4a: Comparison Of Bcl2/Bax Between The Age Ranges				
Age ranges	Mean	Std. Deviation	P-VALUE	Sig.
Age 25 to 45	1.0140	.40605	.144	NS
Age 46 to 65	.5533	.06110		
Age 66 to 95	1.0433	.23798		

Table 4b: Comparison Of Bcl2/Bax Between The Habits				
Habit	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Smoking	.8300	.	0.000*	S
Gutkha/ maawa	.5800	.08000		
Trauma	1.2260	.21995		

Table 4c: Comparison Of Bcl2/Bax Between The TNM Stages				
TNM Stage	**Mean**	**Std. Deviation**	**P-VALUE**	**Sig.**
Stage I	1.0000	.	.954	NS
Stage II	.9200	.53740		
Stage III	.8775	.37105		

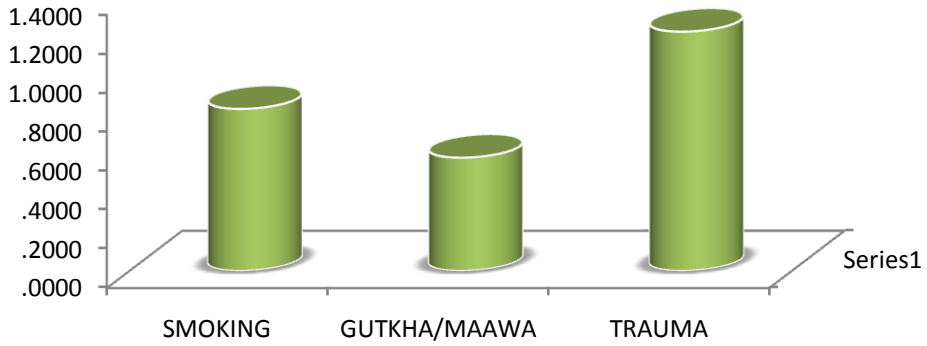

Fig.19: Comparison of bcl2/bax ratio

between the habits (risk factors) of tongue OSCC

DISCUSSION:

Oral cancer is currently the sixth most common type of cancer.[21] The greatest number of OSCC cases is from the south central asian countries which includes India[1]. In India, it is currently the most common cancer type in men (22.9%) and third most common cancer type in women (9.8%) [2].

It affects the age group between 50 to 55 years and it is more common among males .[25] The common site varies among different studies conducted in different population.[19-31] In India, the commonest site is buccal mucosa as the most common habit observed among Indian population is betal quid chewing.[25] It is a type of tumour that is greatly influenced by habit and lifestyle of people, that varies in different parts of the the world. The habit in turn causes differences in the common site affected, and prognosis of OSCC cases in the various epidemiological studies conducted in different regions. The habits that are associated with OSCC include betal quid chewing, maawa/gutkha chewing, snuff dipping, smoking and alcoholism.[25-31]

Oral cancer is not only the common cancer in India but is also the type of the tumour that causes the highest number of deaths due to cancer.[2] The prognosis of OSCC depends on various patient factors (Habit, oral hygiene, immune status etc), tumour related factors (anatomic site, tumour size & depth, nodal metastasis, histological differentiation) and also molecular factors (proliferative and apoptotic factors). [54]

This study concentrates on the immunohistochemical apoptotic proteins namely, bcl2 and bax proteins. The bcl2 is the principal antiapoptotic protein and bax is the principal proapoptotic protein that heterodimerizes with bcl2. [25]The ratio between the bax

and bcl2 determines the fate of cell and acts as the threshold step for apopotosis.[55] This ratio of bcl2/bax is the strongest prognostic marker for OSCC[18] and varies with histological degree of differentiation.[16]

In this prospective study, bcl2/bax is assessed in relation to various clinicopathological factors like age, sex, site of lesion, habit of the patients. During this period the number of cases involving the floor of the mouth (1 case), palate (2 cases) and lips (2 cases) were minimal with no comparable amount of data. Therefore they were eliminated from the study. Among the remaining cases, the first 60 were included in the study.

Majority of the patients were between 45 to 65 years of age (55%). 65% of them were males and 35% were females. Both these observations are in unison with many other studies that were performed earlier.[19-31]

The buccal mucosa was the most common site and formed 30% (18/60 cases), this was followed by alveolar mucosa (26.67% or 16/60 cases), retro molar zone (21.67% or 13/60 cases) and tongue (21.67% or 13/60 cases). This observation coincided with the other sherin et al, Sharma et al etc that was conducted in different parts of India[20, 22, 25, 26]. Although it however differed from other studies conducted elsewhere.[30]

All of the patients had a history of atleast one habit, and the gutkha / maawa chewing habit was the most common habit among the cases (35% i.e 21/60 cases), followed by betal quid chewers (30% or 18/60 cases), smokers (16.67% or 10/60 cases), snuff users (10% or 6/60 cases) and 8.3% of patients had history of trauma (5/60 cases).

All of them who had history of trauma belonged to the tongue OSCC cases. This was also similar to Sharma et al, Sherin et al.[20, 22]

Among the 60 cases studied, 57 cases (95%) were of well differentiated OSCC, and 7 cases are of (11.67%) were of moderately differentiated type. Not a single case of poorly differentiated OSCC was recorded during the period. Since the histological differentiation is known to influence the bcl2/bax ratio[16], those 7 moderately differentiated cases were separated from the rest of the well differentiated cases to avoid the influence of the histological differentiation as a confounding factor.

The difference between the bcl2/bax was not significant between the OSCC cases that involved buccal mucosa, alveolar mucosa and retromolar zone. But the bcl2/bax was significantly different between the tongue cases and the cases that involved other sites. The subsites were then classified according to the age groups, sex, habit and clinical stage. The bcl2/bax values were tabulated accordingly.

Among the buccal mucosa cases, 62.50% were female (10/16 cases) and 37.50% were male (6/16 cases) with a sex ratio of 0.6. This contradicted with the overall sex ratio. The age distribution was however similar to that of the overall picture. The majority of the cases belonged to the 46-65 years group (56.25% or 9/16 cases), with 37.5% in 25-45 years age group (6/16 cases) and 6.25% in the 66-86% age group (1/16 cases). Equal number of cases had betal quid and maawa/gutkha chewing habits (37.5% or 6/16 cases each). This was followed by snuff dipping habit (18.75% or 3/16 cases) and only one case had habit of smoking (6.25%). 50% of cases belonged to the stage IV of TNM

classification. 25 % in the stage III, 12.5% each in stage I and II. None of these factors significantly affected the bcl2/bax ratio.

Among the alveolar mucosa cases, 53.30% were male (8/15 cases) and 46.70% were female (7/15 cases) with a sex ratio of 0.87. This contradicted with the overall sex ratio. The majority of the cases belonged to the 46-65 years group (73.30% or 11/15 cases) and the remaining 26.7% is in 66-95 years age group (4/15 cases). In this group most of the patients had betal quid habit (53.30% or 8/15 cases) followed by smoking and snuff dipping habit (33.3% or 5/15 cases and 13.40% or 2/15 cases respectively). 73.30% of cases belonged to the stage IV of TNM classification, 20% in the stage III and 6.70% in stage I. None of these factors significantly affected the bcl2/bax ratio.

In the retromolar zone cases 91.90% were male (10/11 cases) and 9.10% were female (1/11 cases) with a sex ratio of 0.1. This contradicted with the overall sex ratio. The majority of the cases belonged to the 46-65 years group (54.50% or 6/11 cases) and 45.5% is in 25-45 years age group (5/11 cases). Maximum number of patients had gutka habit (45.5% or 5/11 cases) followed by betal quid habit (27.20% or 3/11 cases) smoking (18.20% or 2/11 cases) and snuff dipping habit (9.10% or 1/11 cases). 72.70% of cases belonged to the stage IV of TNM classification, 18.20% in the stage III and 9.10% in stage I. None of these factors significantly affected the bcl2/bax ratio.

In tongue cases, 91.90% were male (10/11 cases) and 9.10% were female (1/11 cases) with a sex ratio of 0.1. This contradicted with the overall sex ratio. The majority of the cases belonged to the 25-45 years group (45.60% or56/11 cases) followed by 27.20% is in both 45-65 years and 66-95 years age group (3/11 cases in each group). 45.50%

patients had gutka/maawa habit (45.5% or 5/11 cases) and 45.50% had trauma followed by smoking habit (9% or 1/11 cases). 72.70% of cases belonged to the stage III of TNM classification, followed by 18.20% in the stage II and 9.10% in stage I. In this site habits or the risk factors affects the bcl2/bax expression as it shows the significant difference in bcl2/bax ratio between trauma group and smoking and gutkha group (Fig.6).

The statistical significant higher bcl2/bax in the tongue cases infers that the prognoses in these cases are lower than the cases from other subsites. Tongue OSCC cases are known to have poor prognosis than the other OSCC.[56] Various factors have been put forth to explain its poor prognosis.[56] The bcl2/bax might well be a factor that contributes to this fact. Also among the various etiological factors that cause tongue OSCC, trauma forms the most important and common cause of OSCC. The bcl2/bax for the cases due to trauma has the highest value among all other factors. It is not known whether trauma causes a high bcl2/bax ratio or whether an increased bcl2/bax has prompted the development of OSCC when exposed to trauma. However there is a strong correlation between the ratio and trauma. Further studies are required to know more about this.

SUMMARY:

The ratio of bcl2 and bax is an immunohistochemical factor that was already proved to correlate with the histological differentiation and prognosis of OSCC. This study was designed to understand why this ratio is different among different OSCC patients. This was done by assessing the influence of clinicopathological factors like age, sex, site and habit on this ratio and also whether there is difference in the ratio between the different TNM stages.

The prospective was conducted on a sample of first 60 OSCC cases, collected between the month of January 2012 to May 2012, in the Department of Oral and maxillofacial pathology, Tamilnadu government dental college & hospital, Chennai.

During this period the number of cases involving the floor of the mouth (1 case), palate(2 cases) and lips (2 cases) were minimal with no comparable amount of data. Therefore they were eliminated from the study. Among the remaining cases, the first 60 were included in the study. Of the 60 cases, 18 cases involved buccal mucosa, 17 cases involved alveolar mucosa, 14 cases involved retromolar zone, and 13 cases involved tongue. 7 cases were moderately differentiated OSCC and the remaining 53 cases were well differentiated OSCC. There were no poorly differentiated cases. All the patients had a positive habit history for atleast one of the following: Gutkha/maawa chewing, betal quid chewing, smoking, and snuff dipping. Patients who had multiple habits and alcoholism were eliminated. Some of the tongue OSCC cases had a history of trauma and they were also included in the study. The patients' age ranged from 26 to 86 years with

peak incidence in the 45 to 65 years. 65% of the patients were males and 35% were females.

Among the 60 cases 7 of them were moderately differentiated OSCCs. These two classes were separately considered in further evaluation, as the histological differentiation is already known to influence the bcl2/bax ratio and it can be a confounding factor.

The bcl2/bax ratio for the cases were analysed in relation to age, sex, site, for any statistical significance. four subsites i.e; buccal mucosa, alveolar mucosa, retromolar zone and tongue were analysed for statistical significance for. Except for the site of the lesion other factors did not have any statistically significant influence on bcl2/bax. The bcl2/bax for the tongue was significantly higher than the other sites. Therefore the cases were divided into four groups according to the site. Within the groups, the bcl2/bax was assessed according to age groups (25-45 years, 46-65 years, 66-86 years), sex, habit and clinical stages (TNM Stage I, II, III and IV). Among the tongue cases, the ratio varied significantly between the sub-groups according to the habit/etiological factor. Tongue cases who had history of trauma had significantly higher value for bcl2/bax, than the other habits.

CONCLUSION:

This study shows that the ratio of principal anti- and pro- apoptotic regulators i.e, bcl2 and bax in OSCC is significantly high for tongue cases than the other subsites of oral cavity. And within the tongue cases, those that had history of trauma had higher values than the tongue cases with other etiological factors. As bcl2/bax is already known to be inversely proportional to prognosis, this study proves that the bcl2/bax ratio may be a factor for the poor prognosis of tongue OSCC cases. Further studies are required to study the relationship between trauma and bcl2/bax ratio.

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INSTITUTIONAL ETHICAL COMMITTEE
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R.C.No. 0430/DE/2010

Date: 27-03-2012

Title of the Work: " Clinicopathologic Evaluation of Pro- and Anti-apoptotic Marker Expression in Oral Squamous Cell Carcinoma."

Principal Investigator: Dr. B. Prathana, II Year MDS PG Student

Department: Department of Oral Pathology

The request for an approval from the Institutional Ethical Committee (IEC) was considered for the following on the IEC meeting held on 25-01-2012 at the Principal's Chambers, Tamil Nadu Government Dental College & Hospital, Chennai-3.

"Advise to proceed with the study"

The Members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the Principal Investigator.

The Principal Investigator and their team are directed to adhere the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of any change of study procedure, site and investigation or guide.
4. You should not deviate from the area of work for which you have applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions. You should abide to the rules and regulations of the Institution.
6. You should complete the work within the specific period and if any extension of time is required. You should apply for permission again and do the work.
7. You should submit the summary of the work to the ethical committee on completion of the work.
8. You should not claim funds from the Institution while doing the work or on completion.
9. You should understand that the members of IEC have the right to monitor the work with prior intimation.
10. Your work should be carried out under the direct supervision of your Guide/Professor.

SECRETARY 27/3/12

CHAIRMAN

APPENDIX - 1

INFORMATION SHEET

We are conducting a study on **"CLINICOPATHOLOGIC EVALUATION OF PRO- AND ANTIAPOPTOTIC MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA**." For that study, we are selecting patients.

- The purpose of this study is to compare the cell proliferative index and to evaluate the role of causative factors among different sub sites within the oral cavity.
- The identity of the patients participating in the research will be kept confidential throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in the study is voluntary. You are free to decide whether to participate in the study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Name of the patient Signature / Thumb impression

Name of the investigator Signature Date

INFORMED CONSENT FORM

STUDY TITLE:

Clinicopathologic Evaluation of Pro- and Anti-apoptotic Marker Expression in Oral Squamous Cell Carcinoma

Name : O.P.No:

Age / Sex: H.P. No:

Address : S.No:

Tel No :

I, _____ age _____ years exercising my free power of choice, hereby give my consent to be included as a participant in the study, ‘’**Clinicopathologic Evaluation of Pro- and Anti-apoptotic Marker Expression in Oral Squamous Cell Carcinoma’’**

I agree to the following:

- I have been informed to my satisfaction about the purpose of the study and study procedures.
- I hereby give permission to use my medical records for research purpose. I am told that the investigating doctor and institution will keep my identity confidential.
- I agree to share my history of tobacco chewing/smoking habit, and was informed that the institution will keep it confidential.
- I agree for the clinical examination done in this study.
- I am aware that the tissue specimen used in this study is only that which was taken for biopsy and no extra tissue is taken for the study.

Name of the patient Signature / Thumb impression

Name of the investigator Signature Date

APPENDIX - 4

TABLE 1: MASTER CHART

S. No.	OP No.	Age	Site	Gender	Habit	Clinical stage	Differentiation	Bcl2 Sore	Bax Score	Bcl2/Bax ratio
1	14150	70	BM	F	B Quid	IV	Well	3	10	0.3
2	14151	26	T	M	Gutkha/Maawa	II	Well	6	11	0.54
3	14248	30	BM	M	Gutkha/Maawa	IV	Mod	3	9	0.33
4	14250	29	BM	M	Gutkha/Maawa	I	Well	5	9	0.55
5	14257	27	BM	M	Gutkha/Maawa	II	Well	2	9	0.22
6	14265	50	T	M	Gutkha/Maawa	III	Well	6	11	0.54
7	14270	83	T	M	Trauma	I	Well	5	5	1
8	14280	49	RMZ	M	Gutkha/Maawa	IV	Mod	6	5	1.2
9	14284	41	BM	M	Gutkha/Maawa	IV	Well	2	5	0.4
10	14288	41	RMZ	M	Gutkha/Maawa	IV	Well	4	8	0.5

11	14300	55	AM	F	B. Quid	IV	Well	2	11	0.18
12	14302	37	BM	M	Gutkha/Maawa	I	Well	2	5	0.4
13	14312	65	BM	F	B Quid	IV	Well	2	4	0.5
14	14318	50	BM	F	B Quid	IV	Mod	4	7	0.57
15	14327	55	BM	F	B Quid	III	Well	3	10	0.3
16	14328	79	RMZ	M	Gutkha/Maawa	III	Mod	10	7	1.42
17	14329	86	AM	M	B Quid	IV	Well	3	6	0.5
18	14347	35	T	M	Trauma	III	Well	4	3	1.33
19	14350	58	BM	F	B Quid	II	Well	3	10	0.3
20	14359	54	T	M	Gutkha/Maawa	III	Well	5	8	0.62
21	14371	52	AM	F	B. Quid	III	Well	5	10	0.5
22	14375	60	RMZ	F	B Quid	IV	Well	2	4	0.5
23	14383	47	AM	M	Smoking	IV	Well	3	5	0.6
24	14384	45	AM	F	Gutkha/Maawa	IV	Mod	4	7	0.57
25	14386	33	T	M	Trauma	III	Well	3	2	1.5
26	14387	70	AM	F	Snuff	IV	Well	2	6	0.33
27	14395	39	RMZ	M	Gutkha/Maawa	IV	Well	5	10	0.5
28	14396	56	RMZ	M	Snuff	IV	Well	1	2	0.5

29	14398	67	T	M	Smoking	III	Well	5	6	0.83
30	14399	60	BM	M	Gutkha/Maawa	IV	Well	7	11	0.63
31	14407	62	AM	M	Smoking	III	Well	3	8	0.37
32	14410	55	T	M	Smoking	IV	Mod	3	2	1.5
33	14411	55	BM	F	Snuff	IV	Well	2	6	0.33
34	14415	60	RMZ	M	B Quid	IV	Well	4	5	0.8
35	14419	48	BM	F	Snuff	III	Well	5	8	0.62
36	14421	54	T	M	Gutkha/Maawa	III	Well	1	2	0.5
37	14439	50	AM	F	B. Quid	IV	Well	5	8	0.62
38	14440	65	AM	M	Smoking	IV	Well	2	5	0.4
39	14441	58	RMZ	M	Smoking	III	Well	5	6	0.83
40	14444	59	AM	M	B. Quid	IV	Well	2	9	0.22
41	14446	52	RMZ	M	Smoking	IV	Well	2	4	0.5
42	14449	35	T	M	Gutkha/Maawa	III	Well	7	10	0.7
43	14458	75	AM	M	B. Quid	IV	Well	4	6	0.66
44	14460	48	RMZ	M	Gutkha/Maawa	IV	Well	4	8	0.5
45	14461	44	RMZ	M	Gutkha/Maawa	III	Well	4	6	0.66
46	14468	32	T	F	Trauma	III	Well	7	7	1

47	14472	75	AM	F	B. Quid	I	Well	4	6	0.66
48	14473	53	BM	F	B Quid	IV	Well	4	11	0.36
49	14477	62	T	M	Gutkha/Maawa	IV	Mod	3	4	0.75
50	14480	47	AM	F	Snuff	IV	Well	1	2	0.5
51	14497	38	RMZ	M	B Quid	IV	Well	1	1	1
52	14501	55	BM	F	B Quid	III	Well	4	8	0.5
53	14504	41	RMZ	M	Gutkha/Maawa	I	Well	4	12	0.33
54	14507	51	AM	F	B. Quid	IV	Well	3	6	0.5
55	14509	60	BM	F	Snuff dip	IV	Well	4	5	0.8
56	14521	70	T	M	Trauma	II	Well	6	5	1.3
57	14551	45	BM	M	smoking	III	Well	4	8	0.5
58	14556	38	BM	F	Gutkha/Maawa	IV	Well	5	10	0.5
59	14559	53	AM	M	Smoking	III	Well	2	3	0.66
60	14597	60	AM	M	Smoking	IV	Well	5	10	0.5

BM- Buccal mucosa, AM- Alveolar mucosa, RMZ- Retro molar zone, T- Tongue, M- Male, F- female, Well- Well differentiated OSCC, Mod- Moderately differentiated OSCC.

APPENDIX: VI

COMMENTS & SUGGESTIONS OF SUPERVISOR

Dr. Prathana

I have received the files concerning your dissertation on 22-12-2012 through Dr. Baskar (Orthodontics PG). I have gone through it. My comments and suggestions are included below in a tabular format. Contact me as soon as you complete the changes.

Comment 1	1. Principal should be acknowledged first, followed by dissertation screening committee and ethical committee, and then proceed with acknowledging the rest. 2. Remove the word 'guide' where ever necessary and replace with just 'supervisor' 3. I thank Dr. I. Ponniah for his help in the dissertation and overall guidance during my MDS course. 4. I am not the principal investigator. 5. In the declaration by the student, state firmly that I (Dr. Prathana) is entirely responsible for any ethical violations (if any) and it does not have any binding on my supervisor.	Corrections were made as suggested.
Comment 2a	In the abstract section, include aim and objective(s).	Aims & objective included in abstract section
Comment 2b	"Or in other words, all neoplastic evolution requires two important steps, uncontrolled proliferation of cells and suppressed cell death of those cells. The later occurs by a process called apoptosis.[5]"……..verify	The sentence was verified, and the error was identified. Changes made - The cell death occurs by a programmed process called

	the underlined sentence.	apoptosis.
Comment 3a	I am afraid whether the review of literature section is the same as one shown on 15-12-2012? If it is a modified one then show to Dr. Jaikailash and ask him whether it can be written in the present format.	Verified with Dr.Kailash, and the present format was approved.
Comment 3b	"The premise of your dissertation was to evaluate the immunohistochemical expression of bcl-2 and bax in conjunction with clinicopathological parameters to assess their significance in oral SCC." Careful reading of the review of literature section, however, revealed that it does not pertain to the context in question. The aim of a literature review is to demonstrate that thorough knowledge pertinent to a given topic or question is gained or refined by reading the previous literature through critical analysis to explore the gaps or leads in the existing literature, and to relate your study with the previous literature to justify your research intentions. Therefore, the review of literature should be in context with the research objective(s) and also must establish that the literature lacks a similar study or must mention how this study differs with the previous study, if any. I would say that your existing literature search is inadequate and biased. Hence, kindly search relevant literature through electronic and hand search methods, and incorporate appropriately.	The entire format of literature review was changed and and necessary corrections were made.
Comment 4	The scientific content of the review of literature section should be convincing and comprehensible even to a non-pathology dental specialist. It should be written after reading relevant textual content than to write by truncating the abstract section of the	The literature review is re-written in a more descriptive way following the examples given by the supervisor.

	source article. Adopt the following writing method in your literature review section. **1. Jordan et al (2005)** studied the expression pattern of bcl-2 and bax in 30 SCC and observed moderate to intense immunostaining in 60% and 63% of cases for bcl-2 and bax respectively. In this study, the pattern of expression of bcl-2 and bax was inversely related to the grade of the tumor with intense immunostaining for bcl-2 and bax in poorly differentiated and well-differentiated SCC respectively. The authors believed, based on the expression patterns, that alteration in bcl-2 and bax is likely to play a role in the development of SCC, especially during the early stages of epithelial carcinogenesis. **2. Lorro et al (2005)** investigated bcl-2 and bax by immunohistochemistry and in situ hybridization to correlate the expression pattern and to ascertain the mutational spectrum of these genes in epithelial dysplasia and oral SCC. They found that the expression of bax was widely noted in tumor cells of well-differentiated but not in poorly differentiated SCC compared to the low level of expression of bcl-2 in both histological grades of SCC. The authors concluded that loss of bcl-2 in basal cells of epithelial dysplasia and SCC as well as loss of bax in poorly differentiated SCC are not associated with mutations in the coding regions of these genes.	

	3. Incorporate whenever possible the relative expression of markers within the stratum of the lesional epithelium. For example – see below. **Lorro LL et al (1999)** assessed expression of bcl-2 and bax in conjunction with histological grading in oral SCC. They observed a cytoplasmic staining pattern for bcl-2 within the thickness of normal epithelium with intense staining in the basal compartment, but negligible or loss of expression in the basal compartment of well-differentiated SCC. In contrast, bax expression was more intense in the central than in the basal part of the epithelium with higher proportion of bax positive cells in well- and moderately differentiated SCC compared to poorly differentiated SCC. These indicate expression of bcl-2 and bax correlates with the histological grading in SCC.	
Comment 5	The listed studies warrant inclusion in the review of literature section.	
	Staibano S et al. Overexpression of cyclin-D1, bcl-2, and bax proteins, proliferating cell nuclear antigen (PCNA), and DNA-ploidy in squamous cell carcinoma of the oral cavity. Hum Pathol **1998**;29:1189-94.	Included
	Ito T et al. Decreased expression of Bax is correlated with poor prognosis in oral and oropharyngeal carcinoma. Cancer Lett **1999**;140:81-91.	Included
	Homma A et al. Prognostic significance of clinical parameters and biological markers in	Included

	patients with SCC of the head and neck treated with concurrent chemoradiotherapy. Clin Cancer Res **1999**;5:801-806.	
	Lorro LL et al. Apoptosis and expression of Bax and Bcl-2 in snuff and non-snuff associated oral squamous cell carcinomas. Anticancer Res **2000**;20:2855-60.	This article was not available and then on permission from supervisor, it was included in the literature review written with the help of the abstract.
	Teni T et al. Expression of bcl-2 and bax in chewing tobacco-induced oral cancers and oral lesions from India. Pathol Oncol Res **2002**;8:109-114.	Included
	Saeed S et al. Immunohistochemical expression of Bax and Bcl-2 in penile carcinoma. Ann Clin Lab Sci **2005**;35:91-96.	Not included
	Lorro LL et al. Loss of Bcl-2 in the progression of oral cancer is not attributable to mutations. J Clin Pathol **2005**;58-1157-1162.	Included
	Baltaziak M et al. Expression of Bcl-xl, Bax, and p53 in primary tumors and lymph node metastases in oral squamous cell carcinoma. Ann NY Acad Sci **2006**;1090:18-25.	Included
	de Vicente JC et al. Expression of Bcl-2 but not Bax has a prognostic significance in tongue carcinoma. J Oral Pathol Med **2006**;35:140-145.	Included
	Jane C et al. Increased survivin expression in high-grade oral squamous cell carcinoma: a study in Indian tobacco chewers. J Oral Pathol Med **2006**;35:595-601.	Included
	****Coutinho-Camillo CM et al.** Expression of Bcl-2 family proteins and association with clinicopathological characteristics of oral	This aricle was quoted in the indtroduction section and hence was mentioned in the

	squamous cell carcinoma. Histopathology**2010**;57:304-16. **Note:** Although you have referenced this article in the bibliography section, it was not included in the review of literature section.	bibliography section. Now it is also included in literature review section.
	de Sousa FA et al. Comparative analysis of the expression of PCNA, p53, bax, and bcl-2 in oral lichen planus and oral squamous cell carcinoma. Ann Diagn Pathol**2009**;13:308-312.	Included
	Ranganathan K et al. Proliferation and apoptosis markers in oral submucous fibrosis. J Oral Maxillofac Pathol **2011**;15:148-153.	Included
	Zhao J et al. Analysis of thermochemotherapy-induced apoptosis and the protein expressions of bcl-2 and bax in maxillofacial squamous cell carcinoma. Med Oncol**2011**;28:S354-359.	Included
	Jham BC et al. Midkine expression in oral squamous cell carcinoma and leukoplakia. J Oral Pathol Med **2012**;41:21-26.	Included
	Bose P et al. Bax expression measured by AQUA analysis is an independent prognostic marker in oral squamous cell carcinoma. BMC Cancer **2012**;12:332.	Included
Comment 6a	I wish to know, as you have stated in your MM section, that who are those 2 or 3 observers who reviewed the IHC slide for interpretations?	Only one observer reviewed the slides. The corrections are made in the document.

Comment 6b	Annexures should be placed at the back of the document and not at the beginning of the R section.	Adopted
Comment 7	Certain information regarding patient grouping should be mentioned in the MM section and not in the R section.	Adopted
Comment 8	Check the bibliography section for uniformity.	Checked for uniformity.

With regards

Irulandy Ponniah, MDS

Professor and Head

Department of Oral Pathology

TN Government Dental College and Hospital

Chennai 600 003.

APPENDIX - 7

DISSERATION HISTORY & PRE-HISTORY

<table>
<tr><td></td><td></td><td></td></tr>
<tr><td></td><td>Whether a course in research methodology was attended?</td><td>Yes, from 31.01.2011 to 04.02.2011</td></tr>
<tr><td></td><td>Whether adequate training obtained before starting dissertation with regard to conception, design and literature search?</td><td>Yes, it was given in the first year of MDS course.</td></tr>
<tr><td></td><td>When was the dissertation topic selected?</td><td>16.11.2011</td></tr>
<tr><td></td><td>Whether topic selection was discussed with the guide?</td><td>Yes</td></tr>
<tr><td></td><td>Whether a research question put forth?</td><td>Yes</td></tr>
<tr><td></td><td>Whether aim and objective was discussed and got approved from the guide?</td><td>Yes, 02.12.2011</td></tr>
<tr><td></td><td>Whether materials and methods discussed and got approved?</td><td>Yes, 02.12.2011</td></tr>
<tr><td></td><td>Whether the topic got approved from the dissertation screening and ethical committees?</td><td>Yes, 25.01.2012</td></tr>
<tr><td></td><td>Whether the study protocol differed from that initially conceived and approved by the ethical committee? If yes, state the reasons.</td><td>Yes, for analysis of IHC stained tissues manual method is now followed instead of image analysis software as it was not accurate.</td></tr>
<tr><td></td><td>Whether the data for review of literature discussed with the guide?</td><td>No.</td></tr>
<tr><td></td><td>Whether literature review was shown to the guide, and if yes, when it was shown?</td><td>Yes (15.12.2012)</td></tr>
<tr><td></td><td>Who did data analysis and interpretation?</td><td>Myself</td></tr>
<tr><td></td><td>Who did statistical analysis and when it was completed?</td><td>Statistician (18.12.2012)</td></tr>
<tr><td></td><td>Whether bibliography discussed with the guide?</td><td>No.</td></tr>
<tr><td></td><td>Whether printed copy of bibliographies provided to the guide?</td><td>Yes.</td></tr>
<tr><td></td><td colspan="2">When was the following textual content shown to the guide? When was returned to you after correction? Whether the following were approved on initial submission or required number of corrections?
<table>
<tr><td></td><td>Submitted date</td><td>Approved Date</td></tr>
<tr><td>Introduction</td><td>18.08.2012</td><td>23.08.2012</td></tr>
<tr><td>Aims & Objective</td><td>02.12.2011</td><td>02.12.2011</td></tr>
<tr><td>Review of literature</td><td>15.12.2012 & 22.12.2012</td><td>24.12.2012</td></tr>
<tr><td>Materials & Methods</td><td>02.12.2011</td><td>02.12.2011</td></tr>
<tr><td>Results</td><td>22.12.2012</td><td>24.12.2012</td></tr>
</table></td></tr>
</table>

	Discussion	22.12.2012	24.12.2012
	Conclusion	22.12.2012	24.12.2012
	Bibliography	15.12.2012 & 22.12.2012	24.12.2012
	Whether the above sections were edited for language and intellectual content?		Yes
	Whether the final document was checked for overlap with previous work by others?		Checked only for Introduction.
	Whether answer was found to the research question?		Yes